#16

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

DYMECKI

Appln. No. 08/866,279

Filing Date: May 30, 1997

OIPE JC89 MAR 1 4 2000 PATENT & TRADEMARK OFFICE

Group Art Unit: 1632

Examiner: A.-M. Baker

FOR: USE OF FLP RECOMBINASE IN MICE

DECLARATION OF DR. ROBERT E. HAMMER

I, Robert E. Hammer of The University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas 75235-9050, declare the following:

1. I earned a Ph.D. degree in Anatomy & Reproductive Biology from Wayne State University, Detroit, Michigan.

2. I have been a member of the faculty of The University of Texas Southwestern Medical Center for the past 14 years. I am presently Professor of Biochemistry. Prior to this appointment, I was a Postdoctoral Research Fellow with Dr. Ralph L. Brinster at The University of Pennsylvania from 1982 to 1986.

3. I am listed as an author of more than 170 papers on the subject of transgenic animals, a field of research relevant to the production of transgenic mice containing a functional Flp recombinase gene.

4. A copy of my *curriculum vitae* is found under Attachment A.

5. I have no financial interest in any patent resulting from this application, and have nothing personally to gain from issuance of such a patent. A small honorarium will be paid to me by the Carnegie Institution of Washington for my time spent in executing this declaration.

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6. On information and belief, I understand that claims 1-49 are being examined in this application. A copy of the claims as amended is found under Attachment B. The invention claimed in this application is related to a transgenic mouse containing a functional Flp recombinase gene, and its use.

7. On information and belief, I understand that the Examiner has not allowed any of the claims in this application because, *inter alia*, she has concluded they are not patentable over several different combinations of prior art references. Copies of the Office Action dated September 14, 1998; the Response to that Office Action dated January 14, 1999; and the final Office Action dated September 14, 1999 are found under Attachment C. Copies of the references relevant to a sequence-specific recombinase and relied upon by the Examiner are found under Attachment D.

8. To the best of my knowledge, Dr. Susan M. Dymecki (hereinafter Applicant) was the first person to demonstrate that a Flp recombinase transgene could catalyze recombination between Flp-recognition sites in a transgenic mouse. A copy of Applicant's publication dated February 1996 is found under Attachment E. Prior to this demonstration, I would not have expected that making such a transgenic mouse would have been obvious because of prior unsuccessful attempts to demonstrate Flp-catalyzed recombination in a transgenic mouse.

9. It is my opinion that the Examiner's conclusion that claims 1-49 are obvious in view of the prior art is <u>incorrect</u>. Without the benefit of hindsight provided by the successful results obtained by Applicant, a person of ordinary skill in the art would <u>not</u> have had a reasonable expectation of success in making the claimed invention. In particular, the references found under Attachment D do not disclose or suggest transgenic mice as described by claims 1-49, or provide a basis for concluding that a person of ordinary skill in the art would have had a reasonable expectation of

success to make such mice. The facts and reasoning on which my opinion is based is discussed below.

10. I was interested in studying recombination in transgenic mice involving at least one endogenous gene. Preliminary results from this study were published as Palmiter *et al.* (*Banbury Report* 20:123-131, 1985) found under Attachment F.

11. One goal was to control the site of integration of foreign DNA in a trangenic mouse. Although homologous recombination between coinjected DNA molecules had been observed in transgenic mice, recombination between injected DNA and a homologous endogenous gene had not yet been observed.

12. The study was based on my extensive experience with the promoter of the mouse metallothionein-I (*i.e.*, MT) gene, the human growth hormone (*i.e.*, hGH) gene, and the production of transgenic mice. See the list of publications found under Attachment A. The MT promoter can be induced to high levels in transgenic mice by heavy metals like cadmium or zinc. Introduction of a complete hGH gene into a transgenic mouse driven by the MT promoter will produce functional hGH protein that causes the mouse to grow significantly larger. The controls shown on pages 129-130 of Palmiter *et al.* (1985) establish that this provides a robust assay for detecting formation of a complete hGH gene (*i.e.*, recombination) in transgenic mice.

13. Construct #131 is comprised of a MT promoter driving a complete hGH gene and a Flp-recognition site added to the third intron of the hGH gene. Figure 4 shows that a transgenic mouse made with construct #131 grew significantly larger. Thus, insertion of the Flp-recognition site had no effect on MT-hGH expression.

14. Deletion constructs were made. Construct #165 has deleted several hundred basepairs from a region 3' of the Flp-recognition site which is predicted to produce a truncated hGH protein; transgenic mice made with this construct did not grow larger.

In contrast, construct #193 lacks a region 5' of the Flp-recognition site such that the MT promoter and the first two exons of the hGH gene are removed. If fragments #165 and #193 were to recombine in a transgenic mouse at their Flp-recognition sites, a functional hGH protein would be produced from the recombined MT-hGH construct and the transgenic mouse would grow larger.

15. As a control for this study, fragments #165 and #193 were coinjected into fertilized eggs and ten transgenic mice were obtained. Recombination between the coinjected fragments was detected in seven out of ten mice by Southern blotting. Messenger RNA containing the region deleted in construct #165 was detected in five out of ten mice. Two out these transgenic mice grew significantly larger. Note that in this control, recombination occurred between <u>co</u>injected DNA molecules and did <u>not</u> require Flp recombinase. Thus, this control only shows that recombination between fragments #165 and #193 is detectable by increased size of a transgenic mouse, instead of having to detect the complete gene or transcript by laborious techniques. Here, recombination was not dependent on Flp-recombinase activity.

16. Recombination involving at least one endogenous gene and catalyzing such recombination with Flp recombinase were not described in Palmiter *et al.* (1985). Instead, we stated therein, "The ultimate experiment will be to supply the correct information to replace the deleted nucleotides by introducing DNA fragment #193 along with FLPase into eggs from mice carrying resident copies of fragment #165." In the following experiments, I describe efforts to demonstrate that Flp recombinase can catalyze site-specific recombination in a transgenic mouse involving at least one endogenous transgene containing a Flp-recognition site.

17. Construct #330 is comprised of the MT promoter driving a Flp recombinase gene. This Flp recombinase gene was provided by Dr. Michael M. Cox, who had

previously shown that Flp recombinase was necessary and sufficient for site-specific recombination in a system where the yeast Flp recombinase and its substrate, the 2-µm plasmid, was transferred to a bacterial cell (*Proc Natl Acad Sci USA* 80:4223-4227, 1983). Cox (1983) is found under Attachment G.

18. Transgenic mice were made by injecting fertilized eggs with construct #165; hemizygous lines were derived from founder transgenic mice. Several copies of construct #165 were integrated into the genome of the transgenic mice and were analyzed by Southern blotting. Each integrated construct #165 would contain a Flp-recognition site. Thus, recombination between those sites would be expected to alter the number of copies of construct #165 and the length of that repeated array of transgenes.

19. Male transgenic mice made with construct #165 were then mated with female mice, and fertilized eggs from the mating were harvested. Construct #330 was injected into those fertilized eggs and double-transgenic mice (*i.e.*, containing both constructs #165 and #330) were identified. Double-transgenic mice contain a repeated array of transgenes, each containing a Flp-recognition site, and a Flp recombinase transgene.

20. Zinc was added to water and fed to double-transgenic mice containing endogenous Flp-recognition sites and the Flp recombinase transgene. Analysis of the double-transgenic mice, either with basal expression of the MT promoter or after induction of the MT promoter by zinc, was performed to detect recombination between Flp-recognition sites. But despite extensive efforts involving different double-transgenic mice, no recombination was detected.

21. Other experiments performed to detect recombination involving at least one Flp-recognition site from an endogenous gene were similarly unsuccessful. Lines

were established from founders made with a MT-hGH-Flp-recognition site construct (the transgene has a 3' deletion in the hGH gene and, thus, transgenic mice were normal size) and mated to produce fertilized eggs. The fertilized eggs, half of which were expected to contain the transgene, were injected with a Flp-recognition site-hGH construct with a 5' deletion in the hGH gene. Recombination between the Flp-recognition sites of the constructs, one integrated into the genome (*i.e.*, endogenous) and another injected into the fertilized egg, was expected to produce a complete hGH gene and functional hGH protein that would cause mice to grow larger. Flp recombinase activity was provided by coinjecting either expression constructs with the Flp gene or Flp enzyme *per se* into the fertilized eggs. But despite extensive efforts over more than a year, no recombination was detected.

22. The above-described series of experiments were designed to determine whether a Flp recombinase transgene could catalyze site-specific recombination between Flp-recognition sites in a transgenic mouse, in which one recognition site is contained in an endogenous gene of the transgenic mouse. Based on the absence of detectable recombination catalyzed by the Flp recombinase transgene, I conclude that Applicant's claimed invention would not have been obvious at the time it was made because a person of ordinary skill in the art would not have had a reasonable expectation of success to make a transgenic mouse containing a <u>functional</u> Flp recombinase gene. Although construct #330 contains a MT promoter driving a Flp recombinase gene, transgenic mice made with that construct are not considered to contain a functional Flp recombinase gene because no recombination was detected.

23. Furthermore, the references found under Attachment D do not provide a basis for concluding that persons of ordinary skill in the art would have had a reasonable expectation of success in making the claimed invention.

24. Therefore, from the facts discussed above, I conclude that claims 1-49 are patentable over the references cited by the Examiner because a person of ordinary skill in the art would not have had a reasonable expectation of success to make the claimed invention (*i.e.*, a transgenic mouse containing a functional Flp recombinase gene) when it was made by Applicant.

25. The undersigned declares that all statements made herein of my personal knowledge are true and that all statements made on information and belief are believed true; and further that these statements were made with the knowledge that any willful false statements are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of this patent application or any patent issuing thereon.

Date: 3-13-2000

Robert E. Hammer

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re PATENT APPLICATION OF

DYMECKI

Appln. No. 08/866,279 Group Art Unit: 1632

Filed: May 30, 1997 Examiner: A.-M. Baker

FOR: USE OF FLP RECOMBINASE IN MICE

* * *

January 14, 1999

RESPONSE

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

Responsive to the Office Action mailed September 14, 1998 (Paper No. 4), consideration of the following remarks is respectfully requested.

Reconsideration and allowance are requested.

Claims 1-49 are pending and have been examined on the merits.

The amendments to the claims find support throughout the disclosure as originally filed. Therefore applicant submits that no new matter has been introduced.

A form PTO-948 has not been received by applicant.

35 U.S.C. 112

Claim 4 was rejected under Section 112, first paragraph, because the specification allegedly "does not reasonably

provide enablement for introducing the Flp-recognition sequences in such a way as to generate a mosaic transgenic mouse wherein at least two diploid cells have different number of Flp-recognition sequences." Applicant traverses.

In a mosaic or chimeric transgenic mouse, cells that have undergone site-specific recombination between Flp-recognition sequences may differ in the number of Flp-recognition sequences they contain because each cell may undergo a different number of Flp-mediated recombination events. See page 20 of the specification.

Stochastic, stage-specific, and/or tissue-specific recombination can be exploited to alter the genotype of a subset of cells within a transgenic mouse. In stochastic recombination, only a subset of cells in each tissue of the transgenic mouse may harbor sufficient Flp activity to mediate recombination. The result could be a mosaic transgenic mouse that bears clones of genetically distinct cells. Moreover, such a mosaic transgenic mouse may allow cell populations related by cell lineage to be traced and fate maps to be constructed. See pages 13-14 of the specification.

As an illustration of this embodiment of the invention, a mosaic transgenic mouse may be used to study competition between wild-type and mutant cells during development and growth of the mouse since its tissues can be mosaic for these two cell types. See Dymecki (Proc. Natl. Acad. Sci. USA, 93:6191-6196, 1996) which was previously submitted in applicant's Information Disclosure Statement and Dymecki and

Tomasiewicz (Dev. Biol., 201:57-65, 1998) which is submitted herewith for examples where Flp-mediated recombination resulted in mosaicism within different transgenic mouse tissues as demonstrated by Southern blot hybridization.

Thus, Flp-mediated recombination in which the number of recombination events among cells in the transgenic mouse is different may give rise to at least two diploid cells that contain different numbers of Flp-recognition sequences in their genomes.

Claims 15, 41-42 and 47 were rejected under Section 112, second paragraph, as being allegedly indefinite. Applicant traverses.

On page 3 of the Office Action, it was alleged that the meanings of the terms "essential gene" and "developmental gene" is unclear. Applicant respectfully disagrees because these terms are well defined in genetics.

An essential gene may be required for viability of an individual cell or organism. For example, a gene encoding an enzyme may be required to perform an essential function in a metabolic pathway. Moreover, adding a functional version of an essential gene as a transgene may be used to rescue a mouse homozygous for a lethal mutation. The alternatives presented by the Examiner are not contradictory, and the meaning of "essential gene" is clear and definite.

Similarly, a developmental gene may be required to control differentiation of an individual cell or development of an organism. Developmental genes may be involved in the

complex, three-dimensional organization of a functional adult organism: for example, genes involved in differentiation, morphogenesis, determination, and/or pattern formation (see, for example, Gillen, ed., Molecular Biology of the Gene, 4th edition, Benjamin/Cummings Publishing Company, Menlo Park, 1987; and Wilkins, ed., Genetic Analysis of Animal Development, 2nd edition, Wiley-Liss, New York, 1993). The alternatives presented by the Examiner are not contradictory, and the meaning of "developmental gene" is clear and definite.

For all of the foregoing reasons, applicant respectfully requests withdrawal of the rejections under Section 112.

35 U.S.C. 102

"A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." M.P.E.P. § 2131 quoting *Verdegaal Bros. v. Union Oil Co. Calif.*, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). The elements must be "arranged as in the claim." *Lindemann Machinenfabrik v. Am. Hoist & Derrick Co.*, 221 USPQ 481, 485 (Fed. Cir. 1984). In contrast, the references cited in the Office Action (Paper No. 4) do not disclose each and every element arranged as in the pending claims.

Claims 1, 2, 4-19, 22-27, 29-36, 41-43, 45 and 48 were rejected under Section 102(b) as being allegedly anticipated by Kilby et al. (1993). Applicant traverses.

On page 5 of the Action, the Examiner noted that "Kilby et al. did not reduce to practice the generation and use of transgenic mice with the FLP recombinase gene and FRT target sequences." Table 1 of the reference shows that Flp-mediated recombination has not been done to the authors' knowledge, even though Flp appears to have been used in more different species than Cre. In the absence of a disclosure that teaches each and every element of the claimed invention (i.e., the claimed transgenic mouse, method, and system) arranged as in the claims, a prima facie case of anticipation has not been made.

Applicant submits that Kilby et al. does not anticipate the claimed invention because this reference does not put the public in possession of the claimed invention.

Claims 1, 2, 4-13, 22-27, 29-33, 41-43, 45 and 48 were rejected under Section 102(b) as being allegedly anticipated by Wigley et al. (1994). Applicant traverses.

On page 7 of the Action, the Examiner noted that "Wigley et al. did not reduce to practice the generation and use of transgenic mice with the FLP recombinase gene and FRT target sequences." In the absence of a disclosure that teaches each and every element of the claimed invention (i.e., the claimed transgenic mouse, method, and system) arranged as in the claims, a prima facie case of anticipation has not been made.

Applicant submits that Wigley et al. does not anticipate the claimed invention because this reference does not put the public in possession of the claimed invention.

The Examiner is requested to consider the arguments made in the next section prior to using Kilby et al. and/or Wigley et al. to make a new rejection under Section 103(a) in a subsequent Office Action.

For all of the foregoing reasons, applicant respectfully requests withdrawal of the rejections under Section 102.

35 U.S.C. 103

Claims 1, 2, 4-13, 15, 22-27, 29-33, 37-43, 45 and 47-48 were rejected under Section 103(a) as being allegedly unpatentable over Lakso et al. (1992), Wigley et al. (1994), Marx (1993), Marshall (1989), and Bieche et al. (1992). Applicant traverses.

Lakso et al. teach the use of Cre recombinase in a transgenic mouse. As acknowledged on page 8 of the Action, while the reference states "it is likely that other recombinases will be useful in directing precise site-specific DNA rearrangements in transgenic animals," the use of Flp recombinase in transgenic mice is not taught. Thus, the Examiner appears to rely on Wigley et al. to suggest the use of Flp recombinase in transgenic mice.

But a careful reading of Wigley et al. shows that they do <u>not</u> suggest the use of Flp recombinase in transgenic mice. The approach suggested on page 586 shows that transgenic mice are to be generated from ES cells that have been modified by Flp-mediated recombination while in culture. The Flp-mediated recombination contemplated by Wigley et al. would occur in

embryonic stem (ES) cells, not in a transgenic mouse as claimed in the present invention. Although contemplated, this proposal was not reduced to practice by Wigley et al.

Moreover, Wigley et al. describe on page 587 two approaches to supply a pulse of Flp activity in ES cells: transfection of the FLP gene under the control of an inducible promoter and transfection of bacterially-produced Flp protein. In either approach, this reference clearly does not teach or suggest that Flp activity be provided from a FLP transgene in a transgenic mouse.

Marx, Marshall, and Bieche et al. are cited for their teaching various oncogenes and tumor suppressor genes. These references do not address the deficiencies discussed above with respect to Lakso et al. and Wigley et al.

Page 9 of the Action alleges that a reasonable expectation of success would have been anticipated because "the Cre-lox system had already been successfully employed to activate an oncogene in a transgenic mouse." It is further alleged that "the FLP recombinase system is analogous to the Cre recombinase system and functions in a manner that is mechanistically identical to the activity of Cre." Applicant submits that results with Cre cannot be so easily analogized to Flp because the two recombinases do <u>not</u> appear to be identical in their enzymatic functions. Rajewsky's group taught that Flp was not as efficient as Cre in catalyzing recombination in ES cells (page 1160 of Gu et al., 1993). Sauer (Curr. Opin. Biotech., 5:521-527, 1994) is submitted

herewith for its statement on page 524 that Flp catalyzes excision less efficiently than Cre in ES cells. Barinaga (1994), which was previously submitted in applicant's Information Disclosure Statement, reported on page 28 that Flp got a bad reputation when several groups tried to use it to make knockout mice because they had trouble getting it to work well in ES cells. In contrast, page 43 of the specification discloses that Flp recombinase expressed according to the invention can achieve efficient recombination in ES cells on an extrachromosomal substrate.

Lasko et al. and Orban et al. disclosed a Cre transgenic mouse in 1992. But a Flp transgenic mouse was not described in a scientific publication until 1996 (the Dymecki paper submitted in applicant's Information Disclosure Statement). In a letter by O'Gorman and Wahl submitted herewith (Science, 277:1025, 1997), the only publication cited for Flp-mediated recombination in transgenic mice is Dymecki (1996). Also submitted for the examiner's consideration are recent publications using the transgenic line described in the present application (Meyers et al., Nat. Genet., 18:136-141, 1998; Minichiello et al., Neuron, 21:335-345, 1998; and Dymecki and Tomasiewicz, Dev. Biol., 201:57-65, 1998), another line from the Berns group (Vooijs et al., Oncogene, 17:1-12, 1998), and a list of investigators who have requested and received Flp-transgenic mice from applicant.

The foregoing evidence shows that there was not a reasonable expectation of success before the present invention

was made, there was a long lapse of time (about four years) between the publications disclosing transgenic mice with Cre and then Flp, applicant was the first to put the public in possession of the claimed invention, and only one other Flp transgenic line has been published more than two and one-half years after applicant's publication.

Finally, applicant submits that one of ordinary skill in the art would not have been motivated to combine the cited references. The motivation stated on page 9 of the Action is that the combination would "generate a transgenic mouse useful for the study of neoplastic transformation, *in vivo*." But this merely states the result that applicant has achieved, and the resultant combination is not rendered obvious unless the prior art suggests the desirability of the combination. See M.P.E.P. § 2143.01 citing *In re Mills*, 16 USPQ2d 1430, 1432 (Fed. Cir. 1990). Here, the cited references do not suggest the desirability of the combination and there was no reasonable expectation of success in making the claimed invention.

Claims 3, 21, 28, 44, 46 and 49 were rejected under Section 103(a) as being allegedly unpatentable over Wigley et al. (1994), Panigrahi et al. (1992), O'Gorman et al. (1991), Wahl et al. (1997), Hartley et al. (1980), and Buchholz et al. (1996). Applicant traverses.

Wigley et al. allegedly teach the "potential" use of the Flp recombinase system in transgenic mice. As discussed above, however, Wigley et al. do not suggest the use of Flp in

transgenic mice but in ES cells. Furthermore, this suggestion was not supported by a working example.

Panigrahi et al., O'Gorman et al., Wahl et al., Hartley et al., and Buchholz et al. are cited for their teaching various different sequences for Flp and FRT. These references were not cited to address the deficiencies discussed above with respect to Wigley et al.

Page 11 of the Action alleges that a reasonable expectation of success would have been anticipated because "the FLP recombinase gene and FRT target sequences had already been used successfully in cultured mammalian cells as well as in transgenic *Drosophila* (as described in the discussion of the Kilby et al. reference)." Applicant submits that results in tissue culture or with transgenic *Drosophila* cannot be so easily extrapolated to use of Flp in transgenic mice. The recombinase activities documented in Table 1 of Kilby et al. suggests asking the question of why Flp transgenic mice were not done to the authors' knowledge if these results could be readily applied in another context (i.e., a transgenic mouse).

The Stewart group's determination of the thermostability of Flp and Cre recombinases suggests a possible answer to this question and an explanation for the failures of others to make the claimed invention prior to applicant's success: a much lower temperature optimum for Flp than Cre. The abstract of Buchholz et al. (1996) states, "FLP is more thermolabile, having an optimum near 30°C and little detectable activity above 39°C . . . . Cre is optimally efficient at 37°C and

above." They go on to disclose that the F70L mutation in a commercially available plasmid containing the FLP gene renders the Flp recombinase even more thermolabile. Buchholz et al. recommend "the use of Cre for applications in mice that require efficient recombination." Submitted herewith is a more recent publication by Buchholz et al. (Nat. Biotech., 16:657-662, 1998) in which their goal as stated on page 657 was to obtain "an improved FLP recombinase that would redress inactivation by temperatures relevant to mammalian systems," in contrast to temperatures relevant to yeast (30°C) and *Drosophila* (25°C) systems in which Flp had been used. Caution is apparently needed if one assumes that recombinase activity in different cellular contexts will be identical because the improved Flp recombinase disclosed by Buchholz et al. (1998) is three- to five-fold better in cultured mammalian cells while it is four- to ten-fold better in *E. coli*.

Finally, the motivation to combine the cited references is stated on page 11 of the Action as "to generate a transgenic mouse useful for *in vivo* genetic manipulation." Again, this merely states the result that applicant has achieved and the resultant combination is not rendered obvious unless the prior art suggests the desirability of the combination. Here, the cited references do not suggest the desirability of the combination and there was no reasonable expectation of success in making the claimed invention.

Claims 1, 12, 15, 20, 24, 43 and 47 were rejected under Section 103(a) as being allegedly unpatentable over Orban et al. (1992) and Wigley et al. (1994). Applicant traverses.

Orban et al. teach the use of the Cre-lox system in transgenic mice. Wigley et al. allegedly teach the "potential" use of the Flp recombinase system in transgenic mice. As discussed above, however, Wigley et al. do not suggest the use of Flp in transgenic mice but in ES cells.

It is alleged on page 13 of the Action, "One would have anticipated a reasonable expectation of success because the analogous Cre-loxP system had already been successfully employed." As discussed above, however, the successful use of the Cre recombinase system in transgenic mice and the existence of the Flp-FRT system only establishes the long-felt need for the present invention but the evidence presented above shows there was no reasonable expectation of success when the present invention was made because of the different levels of recombinase activity for Cre and Flp.

If the Examiner maintains that the pending claims are prima facie obvious, she is encouraged to consider the long-felt need for a transgenic mouse with a functional Flp transgene and the evidence of failure by others to make such a transgenic mouse as secondary factors favoring patentability of the claimed invention. See the attached list of 33 investigators who have requested and received Flp-transgenic mice from applicant as evidence of this long-felt need and the failure of others.

For all of the foregoing reasons, applicant respectfully requests withdrawal of the rejections under Section 103.

Having responded to all objections and rejections contained in the pending Office Action, applicants submit that the pending claims are allowable and an early Notice to that effect is earnestly solicited. If further information is needed, the Examiner is invited to contact the undersigned.

Respectfully submitted,

Cushman Darby & Cushman
Intellectual Property Group of
PILLSBURY MADISON & SUTRO, L.L.P.

By________________________
Paul N. Kokulis
Reg. No. 16,773
Telephone: (202) 861-3503
Facsimile: (202) 822-0944

PNK/GRT
1100 New York Avenue, N.W.
Ninth Floor, East Tower
Washington, DC 20005-3918
Telephone: (202) 861-3000

Enclosed is a copy of the following:
1. Buchholz et al. (1998) Nat. Biotech., 16:657-662.
2. Dymecki and Tomasiewicz (1998) Dev. Biol., 201:57-65.
3. Meyers et al. (1998) Nat. Genet., 18:136-141.
4. Minichiello et al. (1998) Neuron, 21:335-345.
5. O'Gorman and Wahl (1997) Science, 277:1025.
6. Sauer (1994) Curr. Opin. Biotech., 5:521-527.
7. Vooijs et al. (1998) Oncogene, 17:1-12.
8. A list of 33 investigators who have requested and received a Flp-transgenic mouse from applicant.

Attachment A

CURRICULUM VITAE

3/6/2000

Robert E. Hammer

1. Personal Data

Address:	Howard Hughes Medical Institute L1-410A Department of Biochemistry University of Texas Southwestern Medical Center at Dallas 5323 Harry Hines Blvd. Dallas, Texas 75235-9050
Phone: FAX: E-mail:	(214) 648-5026 (214) 648-7607 hammer@utsw.swmed.edu

Date and Place of Birth:	October 15, 1953 Detroit, Michigan
SSN:	384-60-3360

2. Position

1998 -	Professor Senior Associate Howard Hughes Medical Institute Department of Biochemistry University of Texas Southwestern Medical Center at Dallas
1992 - 1998	Associate Professor Senior Associate Howard Hughes Medical Institute Department of Biochemistry University of Texas Southwestern Medical Center at Dallas
1990 - 1992	Assistant Professor Senior Associate Howard Hughes Medical Institute Department of Biochemistry University of Texas Southwestern Medical Center at Dallas
1986 - 1989	Assistant Professor Assistant Investigator Howard Hughes Medical Institute Department of Cell Biology University of Texas Southwestern Medical Center at Dallas
1989	Adjunct Professor Department of Veterinary Physiology and Pharmacology Texas A & M University College of Veterinary Medicine
1983 - 1986	Research Associate University of Pennsylvania School of Veterinary Medicine

3. Education

1982 - 1986	Postdoctoral Fellow University of Pennsylvania School of Veterinary Medicine Postdoctoral Advisor: Dr. R.L. Brinster
1977 - 1981	Ph.D. in Cell Biology and Anatomy Wayne State University, School of Medicine Dissertation Advisor: Dr. J.A. Mitchell Dissertation Title: Effects of Nicotine and 5-Hydroxytryptamine on Conceptus Development and Implantation in the Rat
1975 - 1977	Physiology Department Wayne State University, School of Medicine Reproductive Physiology: Research on Histological Changes in the Rat Epididymis During Aging.
1971 - 1975	B.A. in Biology Kalamazoo College Kalamazoo, Michigan (Foreign Study in Madrid, Spain, 1973)

4. Support

A. Current

1. Source of support: Howard Hughes Medical Institute
Project Title: Gene Expression in Germ Line Transformed Animals.
Annual amount of award: ~ $450,000
Award Period: 6/1/86 - 8/31/89
Award Period: 9/1/89 - 8/31/91
Award Period: 9/1/91 - 8/31/95
Award Period: 9/1/95 - 8/31/97
Award Period: 9/1/97 - 8/31/99
Award Period: 9/1/99 - 8/31/01

2. Source of support: NIH Grant No. DK 53789-01
Protect Title: TGF-β Signaling in Liver Homeostasis
Direct Costs $215,641
Award Period: 4/1/98 - 3/31/01
PI - R.E. Hammer

3. Source: Perot Family Fund
Project Title: Role of Transforming Growth Factor-β Signaling in Cell Proliferation.
Annual amount of award: $108,000
Award Period: 1/1/92 - 12/31/96
Award Period: 1/1/96 - 12/31/99
Award Period: 1/1/00 - 12/31/03
PI - R.E. Hammer

4. Source: Texas Coordinating Board: Advanced Research Program
Project Title: Renal Fibrosis and Anemia: A TGF-β Transgenic Rat Model (0131-1999)
Direct Costs: $145,042
Award Period: 1/1/00 - 12/31/01

B. Previous

1. Source of support: Texas Advanced Technology Program (Biomedicine)
   Project Title: Transgenic Animal Models of Inflammatory Disease
   Direct Costs $145,000
   Award period: 1/1/92 - 12/21/94
   PI - R.E. Hammer
   Co-PI - Joel D. Taurog

2. Source of support: NIH Grant No. RO1 AI 28332
   Project Title: Immune Reactivity Against Organ-Specific MHC Molecules in Transgenic Animals.
   Direct Costs $760,745
   Award period: 7/31/89 - 6/30/94
   PI - James Forman - Microbiology
   Co-PI - R. E. Hammer

3. Source of support: Crohn's and Colitis Foundation of America Research Grant
   Project Title: Characterization of Inflammatory Gastrointestinal Disease in HLA-B27 Transgenic Rats.
   Direct Costs $150,000
   Award Period: 10/1/91 to 9/30/93
   PI - Joel D. Taurog - Internal Medicine
   Co-PI - R. E. Hammer

4. Source of support: Supplement to NIH Grant No. PO1 AR09989
   Project Title: Immunologic Basis of Rheumatic Disease.
   Direct Costs $190,878
   Award Period: 12/1/91 - 8/31/93
   PI - Joel D. Taurog - Internal Medicine
   Co-PI - R. E. Hammer

5. Source of support: North Texas Chapter Research Grant
   Project Title: HLA-B27-Restricted Anti-Shigella T Cell Responses.
   Direct Costs $60,000
   Award Period: 7/1/89 - 6/30/92
   PI - Joel D. Taurog - Internal Medicine
   Co-PI - R. E. Hammer

6. Source of support: NIH Grant No. P30 AR41940-01; SDR award for Skin Research.
   Project Title: Skin Disease Research Core Center
   Project Director: Paul Bergstresser
   Pilot project #5: Transgenic Rats Expressing HLA Cw6
   Direct Costs $75,375
   Award Period: 9/1/92 - 8/31/95
   PI - Joel D. Taurog - Internal Medicine
   Co-PI - R.E. Hammer

7. Source of support: NIH Grant No. P50 AR39169, Project 1 (SCOR in Rheumatoid Arthritis)
   Project Title: Experimental Arthritis and T Cell Response - MHC class II Transgenic Animals.
   Direct Costs $584,266
   Award Period: 9/1/92 - 8/31/97
   PI - Joel D. Taurog - Internal Medicine
   Co-PI - R. E. Hammer
   Program Director: P.E. Lipsky

8. Source of support: NIH Grant No. R01 DK47692
Project Title: A Transgenic Rat Model of Inflammatory Bowel Disease.
Direct Costs $603,484
Award Period: 9/30/93 - 9/29/97
PI - Joel D. Taurog - Internal Medicine
Co-PI - R.E. Hammer

9. Fellowship - F32 DK 09376-02
for Sarah Comerford, Ph.D.
Title: TGF-β in Cell Proliferation.
Award: $63,000
Award Period: 1/1/95 - 12/31/97

10. Source of support: Excellence in Education Fund
Project Title: Development of Murine Embryonic Stem Cells and Homologous Recombination.
Annual amount of award: $20,000
Award Period: 9/1/92 - 8/31/95
Award Period: 9/1/96 - 8/31/97
Award Period: 9/1/97 - 8/31/98
PI - R.E. Hammer

11. Source of support: NIH Grant No. RO1 AR38319
Project Title: Role of HLA-B27 in Experimental Spondyloarthropathy.
Direct Costs $890,625
Award period: 7/1/94 - 6/30/99
PI - Joel D. Taurog - Internal Medicine
Co-PI - R. E. Hammer

5. Memberships

American Association for the Advancement of Science
American Society of Cell Biology
Society for Developmental Biology

6. Teaching Experience

1999 - Developmental Biology
Genetics and Development Graduate Program (Dennis McKearin - Chair)
2nd year required course
Team taught
One lecture and one discussion session

1997 - 1998 Medical Biochemistry (Carole Mendelson - Course Coordinator)
Section 2 -Molecular Biology
Two lectures - Transgenic Mice and Gene Knock-outs

1992 - 1998 Developmental Biology
Genetics and Development Graduate Program (Steve Wasserman - Chair)
2nd year required course; One-half semester
Co-taught with Dennis McKearin
Eight lectures followed by discussion sessions
Comparison of developmental paradigm in mice and flies

1989	Developmental Biology Department of Biochemistry University of Texas Southwestern Medical Center at Dallas Co-taught with Leland Ellis, Flora Katz and Leon Avery
1987 - 1991	Fundamentals of Cell Biology Department of Cell Biology and Neuroscience University of Texas Southwestern Medical Center at Dallas Course Coordinator
1987 - 1990	Advanced Cellular & Molecular Biology Gene Expression University of Texas Southwestern Medical Center at Dallas (team taught)
1987	Medical Genetics University of Texas Southwestern Medical Center at Dallas (team taught)
1982 - 1985	Reproductive Physiology Laboratory University of Pennsylvania School of Veterinary Medicine Laboratory Assistant
1978 - 1981	Gross Anatomy Laboratories Wayne State University School of Medicine Department of Anatomy Graduate Teaching Assistant
1978	Post Baccalaureate Program Wayne State University School of Medicine Instructor in Histology

The following students have trained with me as postdoctoral or doctoral fellows:

Dr. S.L. Jones	Postdoctoral training, 1987 - 1990 Ph.D. from Massachusetts Institute of Technology
Dr. M.K. Short	Postdoctoral training, 1987 - 1989 Ph.D. from Albert Einstein College of Medicine at Yeshiva University
Dr. H. Yanagisawa	Postdoctoral training, 1991 - 1997 M.D., Ph.D. from University of Tsukuba School of Medicine, Japan
Dr. Bang Wan	Postdoctoral training, 1995 - 1996 Ph.D. from Pennsylvania State University
D.E. Clouthier	Doctoral training, 1989 - 1994 B.Sc. from University of New Mexico
Dr. S. A. Comerford	Postdoctoral training, 1994 - Ph.D. from University of London
Dr. M. Shimomura	Postdoctoral training, 1999 - M.D., Ph.D. from Kobe University Medical School
J. Croy	Doctoral training, 1999 - Co-sponsored with Dave Garbers B.Sc. from University of Florida

7. Patents

1996	Transgenic rats and animal models of inflammatory disease. (US# 5,489,742) R.E. Hammer and J.D. Taurog.

8. Professional Awards, Fellowships and Activities

1998	Member of Organizing Committee and Break-out Group Chairman, NIH Rat Model Repository Workshop, Lansdowne, Virginia, August 19-20
1996 -	Scientific Advisory Board of Induced Mutant Resource, Jackson Laboratory, Bar Harbor, ME
1992	Carol-Nachman Prize in Rheumatology, State Capital of Wiesbaden, Germany
1989 - 1995	Scientific Advisory Board, GenPharm International, Inc., Mountain View, CA., Netherlands
1989	USDA Unit Award for Distinguished Service: Application of Gene Transfer to Farm Animals
1983 - 1984	NIH Postdoctoral Fellowship, University of Pennsylvania School of Veterinary Medicine
1977 - 1981	Graduate Assistantship, Wayne State University, School of Medicine

Research

A. Publications

1. Hammer, R. E., Samarian, R., and Mitchell, J.A. (1978). Alterations in the surface morphology of the antimesometrial uterine epithelium of the rat: Effects of ovarian steroid hormones. Scanning Electron Microscopy. 2:701-706.

2. Hammer, R. E., and Mitchell, F.A. (1979). Nicotine reduces embryo growth, delays implantation and retards parturition in rats. Proc. Soc. Exp. Biol. Med. 162:333-336.

3. Card, J. P., Hammer, R.E., and Mitchell, J.A. (1979). Scanning electron microscopic observations of the early stages of implantation in the rat. Micron. 10:211-212.

4. Hammer, R. E., Mitchell, J.A., and Goldman, H. (1981). Effects of nicotine on conceptus development and oviductal/uterine blood flow in the rat. In Cellular and Molecular Aspects of Implantation. Glasser, S. A. B. , Editor. Plenum Press, New York, NY. pp. 439-442.

5. Hammer, R. E., Goldman, H., and Mitchell, J.A. (1981). The effects of nicotine on uterine blood flow and intrauterine oxygen tension in the rat. J. Reprod. Fert. 63:163-168.

6. Hammer, R. E., and Hafez, E.S.E. (1981). Nutrition and reproduction: Animals. In CRC Handbook of Nutritional Requirements in a Functional Context. Vol. 1. Rechicigl, J. Miloslav, Jr., Editor. CRC Press, Inc., Boca Raton, Fl. pp. 397-426.

7. Palmiter, R. D., Brinster, R.L., Hammer, R.E., Trumbauer, M.E., Rosenfeld, M.G., Birnberg, N.C., and Evans, R.M. (1982). Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature. 300:611-615.

8. Mitchell, J. A., and Hammer, R.E. (1983). Serotonin-induce disruption of implantation in the rat: I. Serum progesterone, implantation site blood flow and intrauterine pO2. Biol. Reprod. 28:830-835.

9. Mitchell, J. A., Hammer, R.E., and Goldman, H. (1983). Serotonin-induced disruption of implantation in the rat II. Suppression of decidualization. Biol. Reprod. 29:151-156.

10. Mitchell, J. A., Hammer, R.E., and Goldman, H. (1983). Concomitant reduction in uterine blood flow and intrauterine oxygen tension in the rat following nicotine administration. Adv. Exp. Med. 159:231-241.

11. McKnight, G. S., Hammer, R.E., Kuenzel, E., and Brinster, R.L. (1983). Expression of the chick transferrin gene in transgenic mice. Cell. 34:335-341.

12. Palmiter, R. D., Norstedt, G., Gelinas, R.E., Hammer, R.E., and Brinster, R.L. (1983). Metallothionein-human grown hormone fusion genes stimulate growth of mice. Science. 222:809-914.

13. Brinster, R. L., Ritchie, K.A., Hammer, R.E., O'Brien, R.L., Arp, B., and Strob, U. (1983). Expression of an immunoglobulin gene in the spleen of transgenic mice. Nature. 306:332-336.

14. Mitchell, J. A., and Hammer, R.E. (1983). Ein vergleich der wirkumgen von nikotin und serotonin auf preimplantation sphanomene in der rate. Verh. Anat. Ges. 77:425-426.

15. Hammer, R.E., Palmiter, R.D., and Brinster, R.L. (1984). The introduction of metallothionein-growth hormone fusion genes into mice. In Advances in Gene Technology: Human Genetic Disorders. Vol. 1. F. Ahmad, Black, S., Schultz, J., Scott, W.A. and Whelan, W.J., Editors. ISCU Press, Miami, Fl. pp. 52-55.

16. Hammer, R. E., Palmiter, R.D., and Brinster, R.L. (1984). Partial correction of a murine hereditary growth disorder by germ-line incorporation of a new gene. Nature. 311:65-67.

17. Swift, G. H., Hammer, R.E., MacDonald, R.J., and Brinster, R.L. (1984). Tissue-specific expression of the rat pancreatic elastase 1 gene in transgenic mice. Cell. 38:639-646.

18. Mitchell, J. A., and Hammer, R.E. (1984). Effects of nicotine on blastocyst development prior to implantation by the rat. In Development Neuroscience: Physiological, Pharmacological, and Clinical Aspects. Caciagli, F., Giacobini, E., and Paoletti, R., Editors. Elsevier Science Publishers, New York, NY. pp. 151-155.

19. Hammer, R. E., Palmiter, R.D., and Brinster, R.L. (1984). Expression of metallothionein-growth hormone genes in transgenic mice. In Endocrinology. Labrie, F., and Proulx, L., Editors. Elsevier Science Publishers, New York NY. pp. 650.

20. Low, M. J., Palmiter, R., Hammer, R.E., Brinster, R.L., Goodman, R.H., and Habener, J.F. (1984). Processing of prosomatostatin expressed by a metallothionein-somatostatin fusion gene in transgenic mice and cultured fibroblasts. In Endocrinology. F. Labrie, and Proulx, L., Editors. Elsevier Science Publishers, New York, NY. pp. 651-652.

21. Low, M. J., Goodman, R.H., Brinster, R.L., Palmiter, R.D., Hammer, R.E., and Habener, J.F. (1984). High plasma levels of immunoreactive somatostatin in transgenic mice expressing a metallothionein-somatostatin fusion gene. Trans. Assoc. Amer. Phys. 97:205-209.

22. Wall, R. J., Pursel, V.G., Hammer, R.E., and Brinster, R.L. (1985). Development of porcine ova that were centrifuged to permit visualization of pronuclei and nuclei. Biol. Reprod. 32:645-651.

23. Ornitz, D. M., Palmiter, R.D., Hammer, R.E., Brinster, R.L., Swift, G.H., and MacDonald, R.J. (1985). Specific expression of an elastase-human growth hormone fusion gene in pancreatic acinar cells of transgenic mice. Nature. 313:600-602.

24. Mitchell, J. A., Hammer, R.E. (1985). Effects of nicotine on oviductal blood flow and conceptus development in the rat. J. Reprod. Fert. 74:71-76.

25. Palmiter, R. D., Hammer, R.E., and Brinster, R.L. (1985). Expression of growth hormone genes in transgenic mice. In Genetic Manipulation of the Early Mammalian Embryo. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp. 123-132.

26. Storb, U., Ritchie, K.A., Hammer, R.E., O'Brien, R.L., Manz, J.T., Apr, B., and Brinster, R.L. (1985). Expression of a microinjected immunoglobulin k gene in transgenic mice. In Genetic Manipulation of the Early Mammalian Embryo. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp. 197-210.

27. Tilghman, S. M., Scott, R.W., Vogt, T.F., Krumlauf, R., Hammer, R.E., and Brinster, R.L. (1985). Tissue-specific expression of cloned α-fetoprotein genes in teratocarcinoma cells and mice. In Genetic Manipulation of the Early Mammalian Embryo. Cold Spring Harbor Laboratory Press,

Cold Spring Harbor, NY. pp. 21-30.

28. Krumlauf, R., Hammer, R.E., Tilghman, S.M., and Brinster, R.L. (1985). Developmental regulation of α-fetoprotein genes in transgenic mice. Mol. Cell Biol. 5:1639-1648.

29. Low, M. J., Hammer, R.E., Goodman, R.H., Habener, J.F., Palmiter, R.D., and Brinster, R.L. (1985). Tissue-specific post-translational processing of preprosomatostatin encoded by a metallothionein-somatostatin fusion gene in transgenic mice. Cell. 41:211-219.

30. Hammer, R. E., Brinster, R.L., Rosenfeld, M.G., Evans, R.M., and Mayo, K.E. (1985). Expression of a human growth hormone-releasing factor gene in transgenic mice results in increased somatic growth. Nature. 315:413-416.

31. Hammer, R. E., Pursel, V.G., Rexroad, C.E., Jr., Wall, R.J., Bolt, D.J., Ebert, K.M., Palmiter, R.D., and Brinster, R.L. (1985). Production of transgenic rabbits, sheep and pigs by microinjection. Nature. 315:680-683.

32. Ebert, K. M., Hammer, R.E., and Papaioannou, V.J. (1985). A simple method for counting nuclei in the preimplantation mouse embryo. Experientia. 41:1207-1209.

33. Hammer, R. E., Brinster, R.L., and Palmiter, R.D. (1985). Use of gene transfer to increase animal growth. In Cold Spring Harbor Symposium on Quantitative Biology. Vol. 50, Cold Spring Harbor, NY. pp. 379-387.

34. Ornitz, D. M., Palmiter, R.D., Messing, A., Hammer, R.E., Pinkert, C.A., and Brinster, R.L. (1985). Elastase I regulated expression of human growth hormone and SV40 T-antigen genes in pancreatic acinar cells of transgenic mice. In Cold Spring Harbor Symposium on Quantitative Biology. Vol. 50, Cold Spring Harbor, NY. pp. 399-409.

35. Krumlauf, R., Hammer, R.E., Brinster, R.L., Chapman, V., and Tilghman, S.M. (1985). Tissue-specific expression of α-fetoprotein genes in transgenic mice. In Cold Spring Harbor Symposium on Quantitative Biology. Vol. 50, Cold Spring Harbor, NY. pp. 371-378.

36. Swanson, L. W., Simmons, D.M., Arriza, J., Hammer, R.E., Brinster, R.L., Rosenfeld, M.G., and Evans, R.M. (1985). Novel developmental specificity in the nervous system of transgenic animals expression growth hormone fusion genes. Nature. 317:363-366.

37. Pursel, V. G., Wall, R.J., Hammer, R.E., and Brinster, R.L. (1985). Cleavage of pig embryos after labeling with fluorescent dyes. Theriogeniology. 24:693-700.

38. Pursel, V. G., Wall, R.J., Rexroad, C.E., Hammer, R.E., and Brinster, R.L. (1985). A rapid whole-mount procedure for nuclei of mammalian embryos. Theriogeniology. 24:687-692.

39. Hammer, R. E., Palmiter, R.D., and Brinster, R.L. (1985). Regulation of growth in transgenic animals. In Regulation and Lactation in Animals. Vol. 1. Schuler, L. A., and First, N.L., Editors. University of Wisconsin Biotechnical Center Series. pp. 51-56.

40. Hammer, R. E., Pursel, V.G., Rexroad, C.E., Jr., Wall, R.J., Bolt, D.J., Palmiter, R.D., and Brinster, R.L. (1986). Genetic engineering of mammalian embryos. J. Anim. Sci. 63:269-278.

41. Krumlauf, R., Chapman, V., Hammer, R.E., Brinster, R.L., and Tilghman, S.M. (1986). Differential expression of AFP genes on the inactive X chromosome in extraembryonic and somatic tissues of a transgenic mouse line. Nature. 319:224-226.

42. MacDonald, R. J., Hammer, R.E., Swift, G.H., Ornitz, D.M., Davis, B.P., Palmiter, R.D., and Brinster, R.L. (1986). Tissue-specific expression of pancreatic genes in transgenic mice. N.Y. Acad. Sci. 478:131-146.

43. Hammer, R. E., Idzerda, R.L., Brinster, R.L., and McKnight, G.S. (1986). Estrogen regulates the avian transferrin gene in transgenic mouse liver. Mol. Cell. Biol. 6:1010-1014.

44. Davidson, B. L., Mathews, L.S., Norstedt, G., Hammer, R.E., Bell, G.I., Brinster, R.L., and Palmiter, R.D. (1986). Expression of growth factor genes in transgenic mice. In Advances in Gene Technology: Molecular Biology of the Endocrine System. D. Puett, Fayal, A., Black, S., Lopez, D., Milna, M., Scott, W., and Whelan, W., Editors. University of Cambridge Press, Cambridge, England. 74-77.

45. Low, M. J., Lechan, R.M., Hammer, R.E., Brinster, R.L., Habener, J.F., Mandel, G., and Goodman, R.H. (1986). Gonadotropin-specific expression of metallothionein fusion genes in pituitaries of transgenic mice. Science. 231:1002-1004.

46. Hammer, R. E., Krumlauf, R., Camper, S., Brinster, R.L., and Tilghman, S.M. (1986). The regulation of α-fetoprotein minigene expression in the germ line of mice. J. Embryol. Exp. Morph. 97:257-262.

47. Welsh, M., Hammer, R.E., Brinster, R.L., and Steiner, D.F. (1986). Stimulation of growth hormone synthesis by glucose in islets of langerhans isolated from transgenic mice. J. Biol. Chem. 261:12915-12917.

48. Quaife, C., Hammer, R.E., and Palmiter, R.D. (1986). Glucocorticoid induction of metallothionein during fetal development of the mouse. Dev. Biol. 118:549-555.

49. MacDonald, R. J., Hammer, R.E., Swift, G.H., David, B.P., and Brinster, R.L. (1986). Transgenic progeny inherit tissue-specific expression of rat elastase I genes. DNA. 5:393-401.

50. Low, M. J., Stork, P.J., Hammer, R.E., Brinster, R.L. Warhol, M.J., Mandel, G., and Goodman, R.H. (1986). Somatostatin secretion is regulated by LHRH in primary pituitary cultures from transgenic mice expressing a metallothionein-somatostatin gene. J. Biol. Chem. 261:16260-16263.

51. MacDonald, R. J., Swift, G.H., Hammer, R.E., Ornitz, D.M., Davis, B.P., Brinster, R.L., and Palmiter, R.D. (1987). Targeted expression of cloned genes in transgenic mice. Adv. Brain Res. 71:3-12.

52. Hammer, R. E., Krumlauf, R., Camper, S., Brinster, R.L., and Tilghman, S.M. (1987). Diversity of α-fetoprotein gene expression in mice is generated by a combination of enhancer elements. Science. 235:53-58.

53. Camper, S. A., Krumlauf, R., Compton, R.S., Hammer, R.E., Brinster, R.L., and Tilghman, S.M. (1987). Expression of the α-fetoprotein gene in transgenic mice. In Gene Transfer Vectors for Mammalian Cells. Miller, J. H., and Callos, M.P., Editors. Cold Spring Harbor Press, Cold Spring Harbor, NY. pp. 110-114.

54. Goldman, M. A., Stokes, K.R., Idzerda, R.I., McKnight, G.S., Hammer, R.E., Brinster, R.L., and Gartler, S.M. (1987). A chicken transferrin gene on the X-chromosome of transgenic mice escapes X-chromosome inactivation. Science. 236:593-595.

55. Ornitz, D. M., Hammer, R.E., Messing, A., Palmiter, R.D., and Brinster, R.L. (1987). Pancreatic neoplasia induced by SV40 T-antigen expression in acinar cells of transgenic mice. Science. 238:188-193.

56. Hammer, R. E., Swift, G.H., Ornitz, D.M., Quiafe, C., Palmiter, R.D., Brinster, R.L., and MacDonald, R.J. (1987). The rat elastase I regulatory elements is an enhancer that directs correct

cell-specificity and developmental onset of expression in transgenic mice. Mol. Cell. Biol. 7:2956-2967.

57. Ornitz, D. M., Hammer, R.E., Davison, B.L., Brinster, R.L., and Palmiter, R.D. (1987). Promoter and enhancer elements from the rat elastase I gene function independently of other inducible and constitutive regulatory elements. Mol. Cell. Biol. 7:3466-3472.

58. Behringer, R., Hammer, R.E., Brinster, R.L., Palmiter, R.D., and Townes, T.M. (1987). Two 3' sequences direct erythroid specific expression of human β globin genes in transgenic mice. Proc. Natl. Acad. Sci. USA. 84:7057-7060.

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63. Pursel, V. G., Rexroad, C.E., Bolt, D.J., Miller, K.F., Wall, R.J., Hammer, R.E., Pinkert, C.A., Palmiter, R.D., and Brinster, R.L. (1987). Progress on gene transfer in farm animals. Vet. Immunol. Immunopathol. 17:303-312.

64. Rexroad, C. E., Pursel, V.G., Hammer, R.E., Bolt, D.J., Miller, K.F., Mayo, K.E., Palmiter R.D., and Brinster, R.L. (1987). Gene insertion: Role and limitations of technique in farm animals as a key to growth. In Biomechanisms Regulating Growth and Development. Steffens, G. L., and Rumsey, T.S., Editors. Kluwer Academic Publishers. pp. 87-97.

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Attachment B

Claims in U.S. Appln. 08/866,279 After Entry of After-Final Amendment

1. A transgenic mouse comprising a Flp transgene integrated in a genome of the transgenic mouse, wherein the Flp transgene is expressed in a cell of the transgenic mouse at a level of recombinase activity sufficient to catalyze recombination between Flp-recognition sequences of the cell.

2. The transgenic mouse according to Claim 1, wherein the genome further comprises a Flp-recognition sequence.

3. The transgenic mouse according to Claim 2, wherein the Flp-recognition sequence is SEQ ID NO:14 or SEQ ID NO:15.

4. The transgenic mouse according to Claim 2, wherein the transgenic mouse contains at least two diploid cells with different numbers of Flp-recognition sequences.

5. The transgenic mouse according to Claim 2, wherein the genome is hemizygous for the Flp-recognition sequence.

6. The transgenic mouse according to Claim 1, wherein the genome comprises at least two Flp-recognition sequences.

7. The transgenic mouse according to Claim 6, wherein the genome comprises at least two chromosomes, each chromosome comprising a Flp-recognition sequence.

8. The transgenic mouse according to Claim 1, wherein the genome further comprises two Flp-recognition sequences in direct repeat orientation.

9. The transgenic mouse according to Claim 1, wherein the genome further comprises two Flp-recognition sequences in inverted repeat orientation.

10. The transgenic mouse according to Claim 1, wherein the genome further comprises a Cre transgene.

11. The transgenic mouse according to Claim 1, wherein the genome further comprises a drug selectable marker transgene flanked by Flp-recognition sequences, wherein the drug selectable marker is excised in cells containing sufficient Flp recombinase activity.

12. The transgenic mouse according to Claim 1, wherein the genome further comprises another transgene flanked by Flp-recognition sequences.

13. The transgenic mouse according to Claim 12, wherein said another transgene is flanked by Flp-recognition sequences in direct repeat orientation.

14. The transgenic mouse according to Claim 12, wherein said another transgene is flanked by Flp-recognition sequences in inverted repeat orientation.

15. The transgenic mouse according to Claim 12, wherein said another transgene is selected from the group consisting of genes controlling differentiation of a cell or development of an organism, genes required for viability of a cell or organism, cytokine genes, neurotransmitter or neurotransmitter receptor genes, oncogenes, tumor suppressor genes, selectable markers, and histochemical markers.

16. The transgenic mouse according to Claim 15, wherein said another transgene is flanked by Flp-recognition sequences in direct repeat orientation.

17. The transgenic mouse according to Claim 15, wherein said another transgene is flanked by Flp-recognition sequences in inverted repeat orientation.

18. The transgenic mouse according to Claim 12, wherein expression of said additional transgene is activated in cells containing sufficient Flp recombinase activity.

19. The transgenic mouse according to Claim 12, wherein expression of said additional transgene is inactivated in cells containing sufficient Flp recombinase activity.

20. The transgenic mouse according to Claim 1, wherein Flp recombinase activity is regulated by a factor selected from the group consisting of chemical, developmental stage, temperature, and tissue type.

21. The transgenic mouse according to Claim 1, wherein the Flp transgene encodes amino acid sequence SEQ ID NO:17 or SEQ ID NO:19.

22. A transgenic mouse comprising a Flp transgene, wherein the Flp transgene is expressed in a cell of the transgenic mouse at a level of recombinase activity sufficient to catalyze recombination between Flp-recognition sequences of the cell.

23. A transgenic mouse comprising a genome which contains a Flp transgene and a Flp-recognition sequence, wherein the Flp-recognition site has undergone Flp-catalyzed recombination.

24. A method of *in vivo* genetic engineering comprising:

(a) providing a transgenic mouse comprising a genome which contains a Flp transgene and at least two Flp-recognition sequences,

(b) expressing the Flp transgene at a level of recombinase activity sufficient to catalyze site-specific recombination in a cell, and

(c) catalyzing recombination between the two Flp-recognition sequences of the cell.

25. The method according to claim 24, wherein site-specific recombination occurs in a germ line cell.

26. The method according to claim 25, further comprising:

(d) mating the transgenic mouse to produce an offspring comprising a recombined genome which does not contain the Flp transgene.

27. The method according to claim 24, wherein site-specific recombination occurs in a somatic cell.

28. The method according to Claim 24, wherein at least one of the Flp-recognition sequences is SEQ ID NO:14 or SEQ ID NO:15.

29. The method according to Claim 24, wherein the genome comprises at least two chromosomes and each chromosome contains a Flp-recognition sequence, whereby recombination between the two Flp-recognition sequences causes chromosomal translocation.

30. The method according to Claim 24, wherein the genome comprises a chromosome and the two Flp-recognition sequences are direct repeats flanking a target sequence on the chromosome, whereby recombination between the two Flp-recognition sequences causes excision of the target sequence.

31. The method according to Claim 30, wherein the target sequence is a drug selectable marker.

32. The method according to Claim 24, wherein the genome comprises a chromosome containing a first Flp-recognition sequence and a target sequence containing a second Flp-recognition sequence, whereby recombination between the two Flp-recognition sequences causes insertion of the target sequence into the chromosome.

33. The method according to Claim 24, wherein the genome comprises a chromosome containing a first Flp-recognition sequence and a plasmid containing a transgene and a second Flp-recognition sequence, whereby recombination between the two Flp-recognition sequences causes insertion of the transgene into the chromosome.

34. The method according to Claim 24, wherein the genome comprises a chromosome and the two Flp-recognition sequences are inverted repeats flanking a target sequence on the chromosome, whereby recombination between the two Flp-recognition sequences causes inversion of the target sequence.

35. The method according to Claim 34, wherein expression of the target sequence is increased by the inversion.

36. The method according to Claim 34, wherein expression of the target sequence is decreased by the inversion.

37. The method according to Claim 24, wherein recombination causes activation of an oncogene or inactivation of a tumor suppressor gene in the cell, thereby transforming the cell and establishing a probability of developing cancer in the transgenic mouse.

38. The method according to Claim 37, further comprising:

(d) administering a candidate agent to the transgenic mouse; and

(e) identifying the candidate agent as a cancer promoter if the probability of developing cancer increases or a cancer inhibitor if the probability of developing cancer decreases.

39. The method according to claim 37, wherein the oncogene is selected from the group consisting of ABL1, BCL1, BCL2, BCL6, CBFA2, CBL, CSF1R, ERBA, ERBB, EBRB2, ETS1, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYCL1, MYCN, NRAS, PIM1, PML, RET, SRC, TAL1, TCL3, and YES.

40. The method according to claim 37, wherein the tumor suppressor gene is selected from the group consisting of APC, BRCA1, BRCA2, DCC, MADH4, MCC, NF1, NF2, RB1, WT1, and TP53.

41. The method according to claim 24, wherein Flp-mediated recombination activates ectopic expression of a gene controlling differentiation of a cell or development of an organism.

42. The method according to claim 24, wherein Flp-mediated recombination inactivates post-embryonic expression of a gene controlling embryonic development of the transgenic mouse.

43. The method according to claim 24, wherein Flp-mediated recombination identifies a cell lineage in the transgenic mouse.

44. The transgenic mouse according to Claim 24, wherein the Flp transgene encodes amino acid sequence SEQ ID NO:17 or SEQ ID NO:19.

45. A system for genetic manipulation, comprising:

(a) the transgenic mouse according to Claim 22, and

(b) a purified nucleic acid comprising a Flp-recognition sequence.

46. The system according to Claim 45, wherein the Flp-recognition sequence is SEQ ID NO:14 or SEQ ID NO:15.

47. The system of claim 45, wherein the purified nucleic acid further comprises a sequence selected from the group consisting of genes controlling differentiation of a cell or development of an organism, genes required for viability of a cell or organism, cytokine genes, neurotransmitter or neurotransmitter receptor genes, oncogenes, tumor suppressor genes, selectable markers, and histochemical markers.

48. The system of claim 45, further comprising:

(c) means for producing a transgenic mouse comprising a genome which contains the Flp-recognition sequence.

49. The system of claim 45, wherein the Flp transgene encodes amino acid sequence SEQ ID NO:17 or SEQ ID NO:19.

Attachment C

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Tradem rk Offic
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Washinoton, DC 20231

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
			234560

HM31/0914

CUSHMAN DARBY & CUSHMAN
INTELLECTUAL PROPERTY GROUP OF
PILLSBURY MADISON & SUTRO LLP EAST TOW
1100 NEW YORK AVE N W NINTH FLOOR
WASHINGTON DC 20005-3918

EXAMINER
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ART UNIT	PAPER NUMBER

09/14/98

DATE MAILED:

DEC 14 1998

DUE

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

PILLSBURY MADISON & SUTRO
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Office Action Summary	Application No. 08/866,279	Applicant(s) Dymecki
	Examiner Anne-Marie Baker, Ph.D.	Group Art Unit 1632

☐ Responsive to communication(s) filed on ______ DEC 14 1998 ______.

☐ This action is **FINAL**.

☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11; 453 O.G. 213.

A shortened statutory period for response to this action is set to expire 3 month(s), or thirty days, whichever is longer, from the mailing date of this communication. Failure to respond within the period for response will cause the application to become abandoned. (35 U.S.C. § 133). Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

Disposition of Claims

☒ Claim(s) *1-49* is/are pending in the application.

Of the above, claim(s) ______ is/are withdrawn from consideration.

☐ Claim(s) ______ is/are allowed.

☒ Claim(s) *1-49* is/are rejected.

☐ Claim(s) ______ is/are objected to.

☐ Claims ______ are subject to restriction or election requirement.

Application Papers

☐ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.

☐ The drawing(s) filed on ______ is/are objected to by the Examiner.

☐ The proposed drawing correction, filed on ______ is ☐ approved ☐ disapproved.

☐ The specification is objected to by the Examiner.

☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

☐ All ☐ Some* ☐ None of the CERTIFIED copies of the priority documents have been

☐ received.

☐ received in Application No. (Series Code/Serial Number) ______.

☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: ______.

☐ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Attachment(s)

☒ Notice of References Cited, PTO-892

☒ Information Disclosure Statement(s), PTO-1449, Paper No(s). 3

☐ Interview Summary, PTO-413

☐ Notice of Draftsperson's Patent Drawing Review, PTO-948

☐ Notice of Informal Patent Application, PTO-152

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--- *SEE OFFICE ACTION ON THE FOLLOWING PAGES* ---

Applicant

Claims 1-49 are pending in the instant application.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claim 4 is rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for integration of Flp-recognition sequences into the genome of a mouse, does not reasonably provide enablement for introducing the Flp-recognition sequences in such a way as to generate a mosaic transgenic mouse wherein at least two diploid cells have different number of Flp-recognition sequences. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

The specification does not provide an enabling disclosure for how to make and use transgenic mice that are mosaic with respect to the integrated Flp-recognition sequences. The specification does not provide any guidance for making a transgenic mouse wherein the cells have different numbers of Flp-recognition sequences integrated into the genome. The specification also does not provide any guidance on how one would use such animals.

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 15, 41, 42, and 47 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 15 and 47 are indefinite in the recitation of the transgene or nucleic acid selected from the group consisting of a developmental gene or an essential gene. It is unclear what is meant by the term "essential gene" because it is not evident in what respect the gene is considered to be essential. The gene could be essential for the viability of the organism or essential for a particular function. Additionally, it is unclear what is meant by a "developmental gene" because such a gene could function in the control of development or could be differentially expressed in different stages of development while not playing a role in the control of development.

Claims 41 and 42 are indefinite in the recitation of a "developmental gene" for the reasons described above.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless --
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1, 2, 4-19, 22-27, 29-36, 41-43, 45, and 48 are rejected under 35 U.S.C. 102(b) as being anticipated by Kilby et al., 1993.

The claims are directed to transgenic mice comprising an Flp transgene and one or more FRT sequences (Flp recognition sequences), wherein the Flp transgene is expressed at a level of recombinase activity sufficient to catalyze recombination between Flp recognition sequences of the cell. The claims are also drawn to a method of *in vivo* genetic engineering wherein a transgenic mouse comprising an Flp transgene and at least two Flp-recognition sequences, expresses the Flp transgene and catalyzes recombination between the two Flp-recognition sequences of the cell.

Kilby et al. teach that site-specific recombinases can be used in transgenic mice to produce an *in vivo* system in which further DNA manipulations can be achieved by selective expression of the recombinase. The reference teaches that the recombinases cre, FLP and R all belong to the λ integrase family of recombinases and show striking similarities in the types of reactions they carry out, the structure of their target sites, and the mechanism of recombination (p. 413, paragraph 3). The orientation of the target sites determines what type of reaction takes place. Recombination between sites in a direct (head-to-tail) repeat causes excision of the intervening DNA as a circular molecule. The reverse of deletion is the targeted integration of circular DNA into the linear molecule; this has potential in gene targeting to a specified chromosomal locus. Recombination between two target sites in an inverted (head-to-head) orientation on the same molecule will invert the DNA between them (page 413, paragraph 5). This mechanism can be exploited to activate or inactivate genes selectively upon induced expression of the recombinase. Both the Cre-lox system and the FLP-FRT system have been used extensively *in vitro* to study gene expression and mechanisms of recombination, as well as to devise strategies for targeted integration of exogenous DNA into the genomic DNA of the cell. The cre-lox system has also been used in transgenic mice and has demonstrated the utility

of the system for activation of a transgene bearing lox target sites (see e.g., p. 417, paragraph 2). The FLP-FRT system has been used in embryonic stem cells to delete an FRT-flanked selectable marker. The FLP-FRT system has also been used *in vivo*, in *Drosophila*. Flies transgenic for a heat shock promoter-FLP construct were crossed to flies whose sole copy of the cell-autonomous eye-color gene *white* was flanked by directly repeated FRT sequences. Deletion of the *white* gene, mediated by FLP expression in both somatic cells and germ line cells, caused white patches in the eye. The extent of mosaicism correlated with the severity and duration of the heat shock. By varying the timing of the heat shock, eye development could be visualized proceeding from the posterior to anterior (p. 418, paragraph 3). Thus, *in vivo* developmental studies have successfully employed the FLP-FRT system. Kilby et al. noted that site-specific recombinases would be useful in transgenic animals for applications in developmental biology, in activating or removing genes at particular stages. Furthermore, they stated that such controlled gene expression could mark a clone of cells for lineage studies, or allow the effect of lethal or deleterious sequences to be studied in a particular cell type or developmental stage (p. 417, paragraph 2). It is noted that although Kilby et al. did not reduce to practice the generation and use of transgenic mice with the FLP recombinase gene and FRT target sequences they provided all of the teachings necessary to enable one skilled in the art to make and use the transgenic mice claimed in the instant invention, including the motivation to use such animals for developmental studies, cell lineage studies, and controlled gene activation/inactivation studies in conjunction with cell-type specific gene expression.

Claims 1, 2, 4-13, 22-27, 29-33, 41-43, 45, and 48 are rejected under 35 U.S.C. 102(b) as being anticipated by Wigley et al., 1994.

The claims are directed to transgenic mice comprising an Flp transgene and one or more FRT sequences (Flp recognition sequences), wherein the Flp transgene is expressed at a level of recombinase activity sufficient to catalyze recombination between Flp recognition sequences of the cell. The claims are also drawn to a method of *in vivo* genetic engineering wherein a transgenic mouse comprising an Flp transgene and at least two Flp-recognition sequences, expresses the Flp transgene and catalyzes recombination between the two Flp-recognition sequences of the cell.

Wigley et al. disclose an approach for accomplishing site-specific transgene insertion using the FLP recombinase system. Their approach seeks to allow single copy insertion of transgenes into a defined site in animal genomes (p. 585, paragraph 4 of Introduction). The method of transgenesis is outlined in the following steps: (1) gene targeting to introduce an FRT site into a specific genomic locus in ES cells, thereby creating the 'transgene acceptor site'; (2) single-copy insertion of transgenes into the targeted FRT site using FLP recombinase; and (3) generation of transgenic animals from the modified ES cells (p. 586, paragraph 6). Wigley et al. contemplate using the FLP recombinase system to excise marker genes from their transfected ES cells. ES cells with an FRT-neo-FRT cassette integrated into the histone H4 gene locus will be transfected with a source of FLP recombinase in order to excise the *neo* gene, thereby generating a single FRT site in the H4 locus, i.e. the transgene acceptor site (TAS) (p. 586, column 2, paragraph 6). FLP recombinase will be used to insert genes, in a single copy, into the TAS. This will involve transfection of the TAS ES cell line with a source of FLP plus a plasmid containing the gene of interest (with regulatory elements) linked to a single FRT. The modified ES cells will then be used to generate transgenic animals (p. 586, column 2, paragraph 7). Wigley et al. indicate that a pulse of FLP activity can be supplied to ES cells by transfecting the FLP gene under the control of the interferon-inducible human '6-16' promoter and that this method has been used successfully to demonstrate FLP-mediated excision (p. 587, paragraphs 4-7). It is

noted that although Wigley et al. did not reduce to practice the generation and use of transgenic mice with the FLP recombinase gene and FRT target sequences they provided all of the teachings necessary to enable one skilled in the art to make and use the transgenic mice claimed in the instant invention, including the motivation to use FLP-mediated recombination to excise marker genes, such as the neomycin resistance gene.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 1, 2, 4-13, 15, 22-27, 29-33, 37-43, 45, 47, and 48 are rejected under 35 U.S.C. 103(a) as being unpatentable over Lakso et al., 1992, Wigley et al., 1994, Marx, 1993, Marshall, 1989, and Bieche et al., 1992.

The claims are drawn to a method of *in vivo* genetic engineering using FLP-FRT transgenic mice wherein recombination causes activation of an oncogene or inactivation of a tumor suppressor gene in the cell, thereby transforming the cell and establishing a probability of developing cancer in the transgenic mouse.

Lakso et al. generated transgenic mice carrying the murine lens-specific αA-crystallin promoter and the simian virus 40 large tumor-antigen gene sequence, separated by a 1.3-kilobase-pair Stop sequence that

contains elements preventing expression of the large tumor-antigen gene and Cre recombinase recognition sites. These transgenic mice were mated with transgenic mice expressing the Cre recombinase under control of the αA-crystallin promoter or the human cytomegalovirus promoter. All double-transgenic offspring developed lens tumors. Analysis confirmed that tumor formation resulted from large tumor-antigen activation via site-specific, Cre-mediated deletion of Stop sequences. Thus the reference teaches the concept of using a recombinase system to activate an oncogene in a specific cell type. Although Lakso et al. do not teach the use of the FLP recombinase system in transgenic mice, they do suggest that the FLP recombinase will be useful in directing precise site-specific DNA rearrangements in transgenic animals, and emphasize that the FLP recombinase of *Saccharomyces cerevisiae* has been shown to be proficient for recombination in both *Drosophila* and in cultured mammalian cells (p. 6235, paragraph 2)

Wigley et al. disclose the potential use of the FLP recombinase system in transgenic mice, as described above. Wigley et al. do not teach the oncogenes disclosed in Claim 39 nor the tumor supressor genes disclosed in Claim 40.

Marshall reviews a number of known oncogenes, including ABL, BCL1, BCL2, ERBA, ERBB, ERBB2, ETS1, FGR, FOS, JUN, MYB, MYC, NRAS, PIM1, SRC, and YES and discusses the mechanisms of oncogene activation. Marshall does not teach the tumor supressor genes contemplated for use in the FLP recombinase-transgenic mice.

Marx discloses a number of known tumor suppressor genes, including APC, DCC, NF1, NF2, RB1, and WT1. As pointed out by Marx, it is well-established in the art that alterations that inactivate tumor suppressor genes play a key role in the development of human tumors. Marx does not discuss the tumor suppressor genes BRCA1, BRCA2, or TP53.

Bieche et al. review the role of the known tumor suppressor genes BRCA1, BRCA2, and TP53 and their mutations in development of breast cancer.

Since the Cre-lox system is analogous to the FLP-FRT recombinase system, and since it would have been desirable to use the eukaryotic FLP-FRT system in mammalian cells in place of the bacteriophage P1 Cre recombinase system, one would have been motivated to substitute the FLP-FRT system for the Cre-lox system, using it in a similar manner to activate oncogenes that have been integrated into the genome of a mouse as specially designed transgene constructs. Since it is well-known in the art that activation of oncogenes and inactivation of tumor suppressor genes both play a major role in neoplastic transformation, one would have been motivated to use the FLP recombinase system for both the activation of oncogenes as well as for the inactivation of tumor suppressor genes in order to study malignant transformation in a particular cell type. One would have anticipated a reasonable expectation of success because the Cre-lox system had already been successfully employed to activate an oncogene in a transgenic mouse. Given the fact that the FLP recombinase system is analogous to the Cre recombinase system and functions in a manner that is mechanistically identical to the activity of Cre, and given that the oncogenes and tumor suppressor genes contemplated for use in the method of Claim 37 are all known in the art, it would have been obvious to one of skill in the art at the time of the invention to have used the FLP recombinase system in a transgenic mouse carrying an appropriate transgene construct comprising FRT sequences such that an oncogene could be activated or a tumor suppressor gene could be inactivated upon expression of the recombinase.

One would have been motivated to have combined the teachings of Lakso et al., Wigley et al., Marshall, Marx, and Bieche et al. in order to generate a transgenic mouse useful for the study of neoplastic transformation, *in vivo*.

Therefore, the claimed invention would have been *prima facie* obvious to one of ordinary skill in the art at the time of the invention.

Claims 3, 21, 28, 44, 46, and 49 are rejected under 35 U.S.C. 103(a) as being unpatentable over Wigley et al., 1994, Panigrahi et al., 1992, O'Gorman et al., 1991, Wahl et al. (US Pat. No. 5,654,182), 1997, Hartley et al., 1980 and Buchholz et al., 1996.

The claims are drawn to a transgenic mouse comprising the Flp transgene and the Flp-recognition sequence, wherein the Flp-recognition sequence is SEQ ID NO:14 or SEQ ID NO:15 and the Flp transgene encodes the amino acid sequence SEQ ID NO:17 or SEQ ID NO:19, and a method of using such a transgenic mouse for *in vivo* genetic engineering.

Wigley et al. disclose the potential use of the FLP recombinase system in transgenic mice as described above under the 102(b) rejection. Wigley et al. do not disclose the amino acid sequence of the FLP recombinase or the DNA sequence of the FRT target sequence.

Panigrahi et al. disclose the DNA sequence of the FRT target sequence referred to as SEQ ID NO:15 in the instant application (see Figure 1).

O'Gorman et al. disclose the DNA sequence of the FRT target sequence referred to as SEQ ID NO: 14 in the instant application (see Reference Note 2 and p. 1351, column 3, paragraph 2).

Either Wahl et al., 1997 (US Pat. No. 5,654,182) or Hartley et al., 1980 disclose the amino acid sequence of FLP recombinase referred to as SEQ ID NO:19. See SEQ ID NO:1 and SEQ ID NO:2 of US Pat. No. 5,654,182 or Figure 2 of Hartley et al.

Buchholz et al. disclose a temperature-sensitive mutant of FLP recombinase wherein the phenylalanine at position 70 is mutated to leucine. This amino acid change, in conjunction with the complete

sequence disclosed by Hartley et al., as more concisely depicted by Wahl et al. (US Pat. No. 5,654,182), corresponds to SEQ ID NO:17.

Since the FLP recombinase system is desirable for use in a transgenic mouse for *in vivo* manipulation of the integrated transgene constructs, one would have been motivated to construct such a transgenic mouse using the amino acid sequence of FLP recombinase and the DNA sequences of the FRT target site, from the information available in the prior art. One would have anticipated a reasonable expectation of success because the FLP recombinase gene and FRT target sequences had already been used successfully in cultured mammalian cells as well as in transgenic *Drosophila* (as described above in the discussion of the Kilby et al. reference). Therefore, it would have been obvious to one of skill in the art at the time of the invention to have used the sequence information available in the literature to construct transgenes for the generation of transgenic mice carrying a functional FLP recombinase gene and FRT target sequences.

One would have been motivated to have combined the teachings of Wigley et al., 1994, Panigrahi et al., 1992, O'Gorman et al., 1991, Wahl et al. (US Pat. No. 5,654,182), 1997, Hartley et al., 1980 and Buchholz et al., 1996 in order to generate a transgenic mouse useful for *in vivo* genetic manipulation.

Therefore, the claimed invention would have been *prima facie* obvious to one of ordinary skill in the art at the time of the invention.

Claims 1, 12, 15, 20, 24, 43, and 47 are rejected under 35 U.S.C. 103(a) as being unpatentable over Orban et al., 1992 and Wigley et al., 1994.

The claims are drawn to an FLP-FRT transgenic mouse wherein the transgene flanked by the FRT target sequences is selected from the group consisting of developmental gene, essential gene, cytokine gene, neurotransmitter gene, neurotransmitter receptor gene, oncogene, tumor suppressor gene, selectable marker,

and histochemical marker. Furthermore, the recombinase activity is regulated by a factor selected from the group consisting of chemical, developmental stage, temperature, and tissue type. Claims 24 and 43 are drawn to a method of *in vivo* genetic engineering wherein Flp-mediated recombination identifies a cell lineage in the transgenic mouse.

Orban et al. disclose the use of the Cre-lox system in transgenic mice wherein the the recombination target transgene is a β-galactosidase gene flanked by *loxP* target sequences. Mice carrying the Cre recombinase transgene under the control of the thymocyte specific *lck* promoter were bred to mice carrying the *loxP*-β-gal-*loxP* transgene construct also under control of the *lck* promoter to obtain doubly transgenic mice. These mice exhibited tissue-specific DNA recombination as a result of Cre expression (p. 6862, paragraph 3 and Figure 2). Southern analysis indicated that the recombination occurred specifically in thymocytes of the doubly transgenic mice and not in tail DNA. The recombined DNA structure also was not present in parental thymocyte DNA from either the Cre or *loxP*-β-gal-*loxP* transgenic mice. Orban et al. also disclose that Cre-mediated recombination provides a heritable marker for mitoses following the loss of Cre expression (Abstract). Although Cre expression was not detected in the splenic T cell subpopulation, a high degree of recombination within the *loxP*-β-gal-*loxP* transgene array was detected, implying that these T cells derived from thymocytes in which recombination had occurred. Thus, the heritable nature of Cre-mediated recombination provides a methodology for cell fate determinations in mammals. This method depends on tissue-limited expression of the recombinase transgene, wherein thymocytes that express Cre recombinase develop into mature T cells that are not capable of expressing Cre recombinase. Although Orban et al. do not teach the use of the FLP recombinase system in transgenic mice, they do emphasize that Cre activity appears mechanistically identical to that of yeast FLP recombinase and that both recombinases have been used in cultured eukaryotic cells and *in vivo* in Drosophila to direct site-specific recombination (p. 6861, paragraph

2). Thus, Orban et al. teach the use of a recombinase system analogous to the FLP system in transgenic mice wherein the transgene flanked by the target sequences encodes a histochemical marker as claimed in the present invention for the FLP system. Furthermore, the recombinase activity is regulated by tissue type due to the use of the *lck* promoter which exhibits thymocyte-specific activity. As in the instantly claimed invention, the recombination event can be used to identify a cell lineage in the transgenic mouse.

Wigley et al. disclose the potential use of the FLP recombinase system in transgenic mice, as described above.

Since the Cre-loxP system is analogous to the FLP-FRT recombinase system, and since tissue-specific expression of genes is a method known in the art to be extremely useful for analysis of gene expression and gene function, one would have been motivated to use regulated expression of marker genes, either histochemical markers or selectable markers, in the context of the FLP-FRT recombinase system in order to achieve high-efficiency recombination in a eukaryotic system with the combined advantage of controlled expression of easily detectable genes and gene products. One would have anticipated a reasonable expectation of success because the analogous Cre-loxP system had already been successfully employed to analyze recombination events and cell lineage in transgenic mice using a histochemical marker gene in a tissue-restricted manner. Therefore, it would have been obvious to one of skill in the art at the time of the invention to have used the FLP recombinase system in a transgenic mouse carrying a marker gene, wherein either or both the marker gene and/or the recombinase gene are under the control of a tissue-specific control element (such as a promoter or locus control region).

One would have been motivated to have combined the teachings of Orban et al. and Wigley et al. in order to develop an *in vivo* system useful for the analysis of cell fate or regulated gene expression, wherein

genes can be selectively turned on or off at will, depending on the type of regulatory control region included in the transgene constructs.

Therefore, the claimed invention would have been *prima facie* obvious to one of ordinary skill in the art at the time of the invention.

No claim is allowable.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Anne-Marie Baker whose telephone number is (703) 306-9155. The examiner can normally be reached Monday through Friday from 8:00 AM to 5:30 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Jasemine Chambers, can be reached on (703) 308-2035. The fax phone number for the organization where this application or proceeding is assigned is (703) 308-4242.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is (703) 308-0196.

Anne-Marie Baker, Ph.D.
September 14, 1998

BRIAN R. STANTON
PRIMARY EXAMINER
GROUP ~~1800~~ 1600

Attachment D

Site-specific recombinases: tools for genome engineering

NIGEL J. KILBY, MICHAEL R. SNAITH AND JAMES A.H. MURRAY

Site-specific recombinases from bacteriophage and yeasts have been developed as novel tools for manipulating DNA both in the test-tube and in living organisms. We discuss the characteristics of these enzyme systems, review their application in genetic and developmental studies and speculate on their future potential for large-scale directed modifications of eukaryotic genomes.

A number of bacterial and yeast elements encode recombinase enzymes that cleave DNA at specific target sequences, and then ligate it to the cleaved DNA of a second site. This simple but elegant reaction results in a precisely defined recombination between two appropriate target sequences. The complexity of these recombination systems varies considerably, both in their requirement for ancillary proteins and in the size of the DNA sites involved[1,2]. However, certain site-specific recombinases, including cre from bacteriophage P1 and FLP from yeast plasmids, are in themselves sufficient to catalyse recombination between specific target DNA sites of around 35 bp. Target sites of this size are unlikely to occur at random in higher eukaryotic genomes and, since the only other requirement for the reaction is expression of the appropriate recombinase, these enzymes offer all the necessary characteristics of a site-specific recombination system for use in any species. Here, we review recent successes in manipulating DNA with site-specific recombinases, both *in vitro* and *in vivo*.

The recombinase systems

Three principal site-specific recombinases have been used to manipulate DNA in heterologous cellular environments. The 38 kDa cre (causes recombination) recombinase of bacteriophage P1 recognizes a 34 bp site called *loxP* (locus of crossing-over). Its natural environment is the circular plasmid form of the P1 phage, which carries a single copy of *loxP* per genome unit[3,4]. The main function of cre is to ensure stable maintenance of the plasmid by resolving DNA dimers into plasmid monomers (Fig. 1a). The 43 kDa FLP (pronounced 'flip') recombinase is encoded by the 2μ circular plasmid of budding yeast, *Saccharomyces cerevisiae*[5,6]. This plasmid has two 34 bp recombinase target sites, arranged in an inverted orientation (Fig. 1b). Recombination between these FRT (FLP recognition target) sites results in inversion of one half of the plasmid with respect to the other. This provides a mechanism for producing multiple plasmid copies from a single replication initiation by flipping the direction of migration of one replication fork[7]. The third recombinase, R, is encoded by the pSR1 plasmid of the yeast *Zygosaccharomyces rouxii*, where it performs a role similar to FLP (Refs 8, 9).

Recombination events

cre, FLP and R all belong to the λ integrase family of recombinases and show striking similarities, not only in the types of reaction they can carry out, but also in the structure of their target sites and mechanism of recombination (Figs 2, 3).

These recombinase enzymes can interact with target sites on a variety of substrates. Recombination between sites in a direct (head-to-tail) repeat on a circular molecule excises the intervening DNA and generates two circular molecules (Fig. 1a), whereas recombination between sites on separate molecules reverses the resolution reaction, and produces a cointegrate. Recombination between target sites in an inverted (head-to-head) orientation inverts the intervening DNA (Fig. 1b).

Since supercoiling of the DNA substrate is not required for recombinase activity, reactions can also occur between sites on linear molecules (Fig. 4). Recombination between two inverted sites on the same molecule will invert the DNA between them (Fig. 4a), while directly repeated targets cause excision of intervening DNA as a circular molecule (Fig. 4b). The reverse of deletion is the targeted integration of circular DNA into the linear molecule; this has potential in gene targeting to a specific chromosomal locus. Finally, if targets are present on separate linear molecules, recombinase action will result in mutual exchange of regions distal to the site (Fig. 4c). Here, the potential for chromosome engineering is apparent, and could be exploited to allow reciprocal exchange of chromosome arms.

Reversibility

These recombinations occur with base-pair precision, so active sites remain afterwards (Fig. 2). Each recombination is therefore reversible, and its products represent an equilibrium of the forward and backward reactions. This may not be a significant problem when DNA fragments are being deleted (Fig. 4b), since excision is an intramolecular event and is therefore favoured over integration. Moreover, the small circular molecule formed probably lacks sequences necessary for DNA replication and would be quickly lost *in vivo*. However, when co-integration is desired, it may be necessary to limit recombinase activity to prevent the integrated molecule from being re-excised. This can be achieved by transient expression of the recombinase, either from an inducible promoter[10–15], or by introducing a non-selected plasmid that expresses the recombinase and is subsequently lost[16–21]. Reversibility can also be overcome by using purified recombinase enzyme, which provides a transient source of activity; this approach has been recently demonstrated with cre[22] and could be more widely applied since cre enzyme is now available commercially.

Two other strategies might overcome this problem of reversibility. First, the sequence of the target sites can be manipulated to enhance recombination in a single (forward) direction (Fig. 5a). Second, in an approach being developed in several laboratories, one of the target sites can be placed between the recombinase gene and its promoter, so that the desired reaction separates the gene

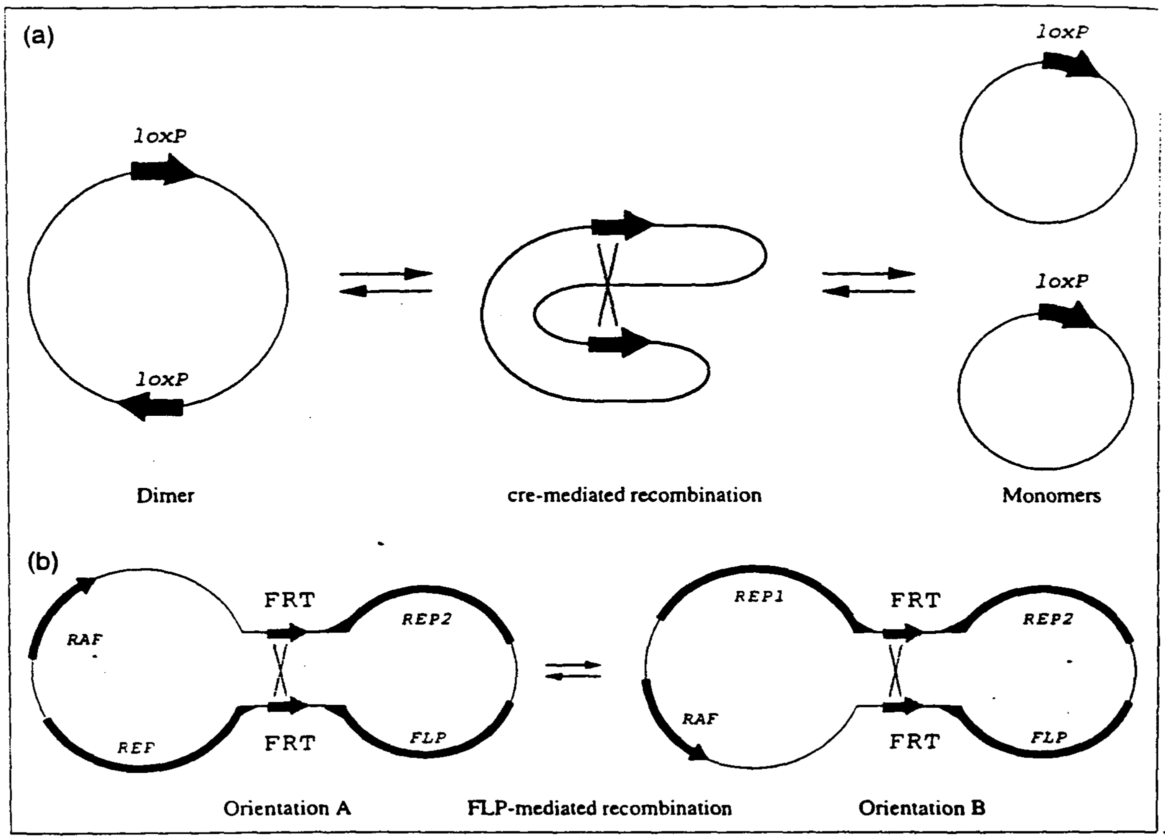

FIG 1

Functions of site-specific recombinases and alignment of target sites. (a) As part of its life cycle, bacteriophage P1 replicates as a single-copy circular plasmid in *E. coli*. The site-specific recombinase cre resolves plasmid dimers into monomers, increasing the number of segregating molecules and preventing plasmid loss[3]. cre acts on the 34 bp site *loxP* (filled arrows); each circular genome has a single *loxP* site. Dimers have two *loxP* sites, both oriented in the same direction (directly repeated); recombination between these sites excises DNA lying between them. Recombination can also occur between two monomers, leading to formation of a co-integrate, but this intermolecular reaction is generally less favoured than intramolecular excision. (b) FLP is encoded by the 2μ circle, a high-copy-number nuclear plasmid of *S. cerevisiae*[5]. The plasmid also encodes three other genes required for its maintenance, *REP1*, *REP2* and *RAF*, but these are not involved in FLP recombination. FLP converts the plasmid between the two isomers shown by acting on target FRT sites (shaded arrows), which are inverted with respect to each other. The FRTs are located within much larger inverted repeat (IR) structures of 599 bp (parallel horizontal lines); this 'dumb-bell' representation of 2μ circle is conventionally used to indicate the homology of these IRs. The biological role of FLP is to provide a mechanism for producing multiple plasmid copies from a single initiation of DNA replication[7]. A number of related yeast species contain plasmids similar to 2μ in structure and maintenance, including pSR1, a plasmid of the osmotolerant yeast *Zygosaccharomyces rouxii*. This encodes the R recombinase, which acts on short specific targets[9] found within large inverted repeats of 959 bp (Fig. 3).

from its expression signals (Fig. 5b). A more sophisticated approach would be to link silencing of the recombinase with activation of a previously promoter-less selectable marker gene; this would be particularly useful in targeted integration, since random integration of incoming DNA would fail to activate the reporter gene.

Cloning tools

With appropriate positioning of target sites, recombinase systems should allow the experimenter to invert, delete, insert or translocate DNA molecules. The discovery that FLP is active not only in its native yeast but also when expressed in *E. coli*[23], and that cre could function when introduced into yeast[24], showed that host-specific functions were not required for their activity and opened the way for their development as useful tools for various cloning applications. Familiar to many molecular biologists is the automatic subcloning of inserts as replicating plasmids from λ phage vectors such as λZAP by infection into bacteria expressing cre[25]. In these vectors, which are largely designed for cDNA cloning, directly repeated *lox* sites flank a region that includes cloning sites, a plasmid origin and a selectable marker. On passage of such vectors through a cre-expressing strain, DNA between the *lox* sites is excised as a circular replicating plasmid molecule.

Cell-free systems that incorporate purified cre recombinase also have their uses, and can simplify introduction

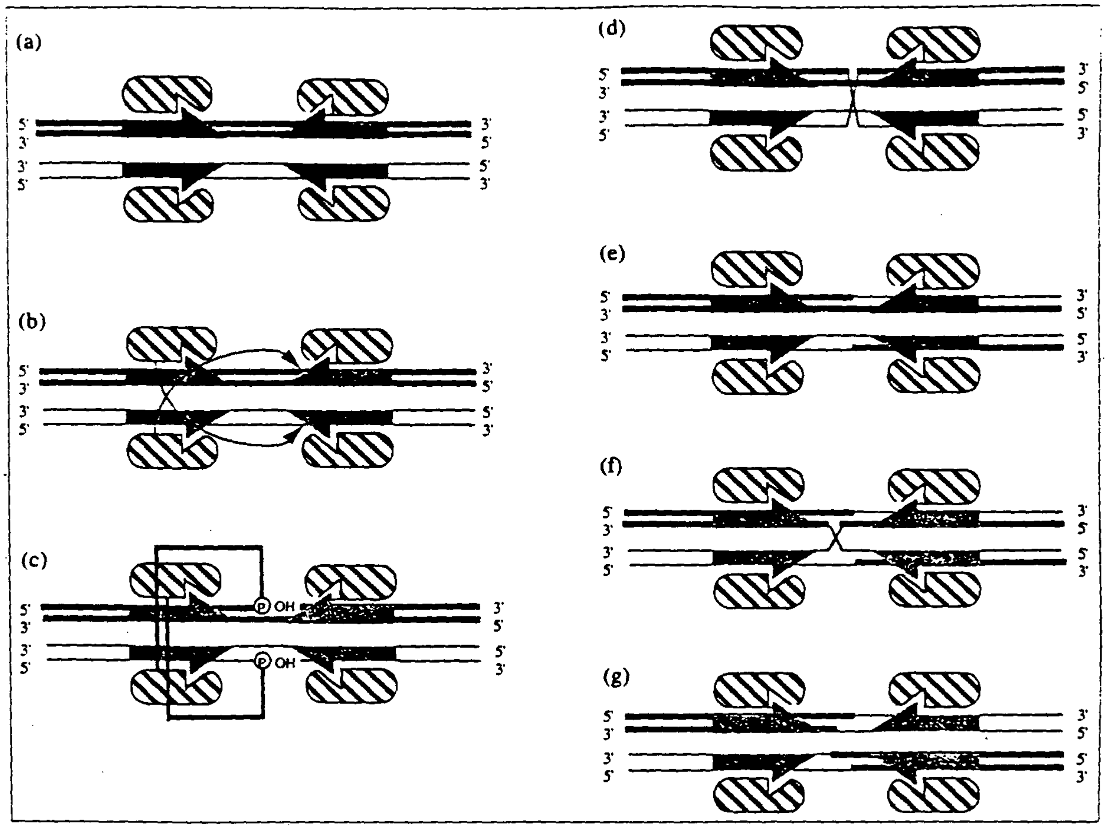

FIG 2

Diagrammatic representation of recombinase action. Catalytic processes of site-specific recombination have recently been reviewed[1]; we cover only those aspects necessary to our discussion. The essential target (filled arrows) for all three proteins consists of two inverted repeats (shaded half-arrows) of 13 bp (12 bp for R) flanking a core sequence of 7 or 8 bp, depending on the recombinase (Fig. 3). Most is known about the FLP-mediated reaction, but reactions catalysed by cre and R probably proceed by very similar routes. (a) A single recombinase molecule binds to each of the inverted repeats so that when synapsis occurs between the two target sites, four protomers are involved. (b) DNA cleavage, probably on the opposite strand to that where the monomer is bound, leads to covalent linkage of a specific tyrosine residue in the FLP monomers to the 5' phosphate of the DNA (c). Strand ligation (d) leads to formation of a Holliday intermediate, here shown in a linear representation (e). The sequence of events is repeated between the other pair of strands (f), to give the final recombination product (g). No divalent cations or high-energy co-factors are required at any stage of the reaction.

of genes into large viral genomes, whether linear or circular. In conventional procedures, genes are cloned into a small transfer vector; this is then introduced into infected cells where it homologously recombines into the endogenous virus at low frequency. Identifying recombinant virus against the wild-type background can be difficult. The recombinase-mediated system requires one *lox* site in the virus genome and a second in the transfer plasmid. Treating the purified viral genomic DNA and transfer plasmid with purified cre recombinase *in vitro* leads to their co-integration and, if cells are re-infected with this product, recombinant can be recovered with high efficiency (20–50%). Moreover, the process can be reversed and the insert isolated as a replicating plasmid. Suitable target viral genomes have been constructed in herpes viruses[26] (100–250 kb) and baculoviruses[27] (80–153 kb).

DNA constructs often contain sequences that, although necessary at an earlier stage of their assembly, later become undesirable. Such regions can be marked for removal by flanking with target sites in the same orientation (Fig. 4b). FLP has been used to remove all *E. coli* sequences from yeast shuttle vectors after their transformation into yeast, increasing both the stability of the yeast plasmid and the acceptability of manipulated organisms for uses in the food and pharmaceutical industries[28]. In a similar vein, FLP was used to eliminate a selectable marker gene from a construct stably integrated into a chromosome of the yeast *Pichia pastoris*, allowing repeated use of the same marker gene[29].

Cellular and transient studies

Perhaps the most exciting application of recombinases is their use *in vivo*. Transient introduction of both target DNAs and plasmids that express the appropriate recombinase has helped assess the potential of these various systems in mediating recombination in the cellular environment of higher eukaryotes. Studies in

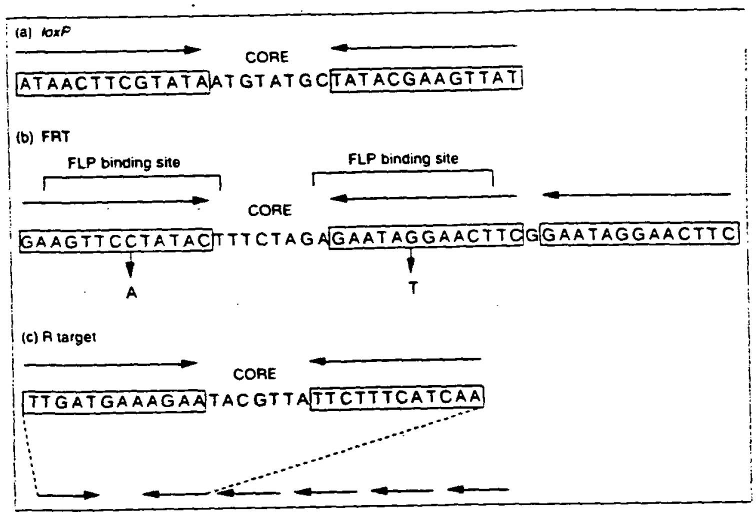

FIG 3

Sequence and structure of target sites: (a) the *loxP* site, target for cre; (b) the FRT site, target for FLP; (c) the target for R. Recombinases bind to the 12–13 bp inverted repeat elements (boxed) that flank the central core region. The core sequence is not involved in recombinase binding, but is the site of crossing-over. The asymmetry of the core gives directionality to the target sites, which can therefore align productively in only one orientation[6]. The core region is also involved in DNA recognition between targets: its sequence can be altered within certain constraints[6,44] to give sites that recombine efficiently with a second identical site, but not with a wild-type target. (b) The 'minimal' 34 bp FRT site is flanked by a third binding site that has a single nucleotide change: the 'wild-type' site is therefore 48 bp. This third binding site appears to enhance the efficiency of the FLP reaction in *E. coli*[45], and may affect the order of cleavage and exchange events during recombination *in vitro*[46]. However, the minimal site can be as effective as the wild-type FRT *in vitro*. Further complexity is added by the possibility of using not just the FRT site, but the complete 599 bp repeat of 2μ plasmid that contains the FRT: this seems to enhance further the intermolecular (co-integration) reaction rate in *E. coli* and yeast[45]. Mutations shown in the 13 bp repeat elements are discussed in the legend to Fig. 5. (c) The binding site for the R recombinase consists of 12 bp inverted repeats flanking a 7 bp core[9]. Four additional directly repeated binding sites for R flank the right-hand minimal site and may stimulate the efficiency of the R-mediated recombination reaction[47].

cells from a variety of species demonstrate that all three recombinases can efficiently excise DNA between two target sites in direct orientation. Inversion between targets in inverted orientation can also occur, as can co-integration of two separate plasmids that each carry a target site, albeit at a lower efficiency (Table 1). Recombination between target sites depends on the presence of recombinase, and the extent of recombination is proportional to the level of recombinase expression. This conclusion is supported by the enhanced cre recombination frequencies seen when translation of cre message is optimized[15] or when a nuclear-targeting signal is added[21].

Experiments in which a target site is integrated into a specific chromosomal location are particularly relevant to the development of transgenic systems. Deletions can occur when integrated constructs containing *lox–lox* are exposed to a transient source of cre recombinase[15,30], while FLP recombinase can efficiently remove a selectable *neo* gene flanked by FRT sites in direct orientation from the genomes of several mammalian cell types[17]. The single FRT left behind by such an excision need not affect expression even when located between a gene and its promoter[17]. In plants, transiently expressed pSR1 R can recognize target sites in constructs integrated in the genome of tobacco cells[18].

Such experiments convincingly demonstrate that site-specific recombinases function efficiently in a wide variety of species and that, at least at certain chromosomal locations, the higher order structure of eukaryotic chromatin does not prevent their action. This raises the possibility of activating genes by removing a blocking sequence[17,18,30], or of eliminating gene activity by deletion[17].

Targeted integration

Although homologous recombination between incoming DNA and the equivalent chromosomal sequences can be used to make targeted alterations to the genomes of mammalian and plant cells, it is inefficient and requires screening for these cells against a background of random integrations. Site-specific recombinase systems have therefore been explored as a way to target incoming DNA to a previously inserted target site, making use of the co-integration reaction (Fig. 4b). Such targeting to a 'docking site' might be particularly valuable when dealing with species in which even non-homologous integration is a rare event, and could increase recovery of stable transformants[14,31].

Studies designed to investigate the FLP-mediated integration of non-replicating λ DNA into FRT sites in the *E. coli* chromosome[32] suggest that the main problem with this type of system is stabilizing the integrated DNA against FLP-mediated excision. Stable integration could only be achieved by switching off FLP expression soon after introduction of the targeted DNA. A similar effect was produced in mammalian cells by transiently expressing FLP from a plasmid cotransfected with the targeted plasmid[17]. Remarkably, in the absence of specific selection for targeted insertion, 60% of clones recovered in this experiment showed FLP-mediated targeting to a resident FRT site (Fig. 4b), suggesting that the problem of re-excision can be overcome[17].

The cre–*lox* system can also target incoming DNA bearing a *lox* site to a chromosomal target site in either mammalian cells or yeast[15]. Repeated targeting to the same site produces subclones in which transgenes are reproducibly expressed[33]; this straightforward and efficient targeting allows the analysis of the expression of numerous constructs integrated into the same chromosomal location, without differences caused by flanking sequences or position effects.

Embryonic stem cells

The potential of site-specific recombinases for DNA manipulation in mammals can only be fully realized by their use in murine embryonic stem (ES) cells. Recently, cre recombination has been used in ES cells in a two-step process that first flanked the J_H–E_μ switch region of an immunoglobulin H gene with *lox* sites using conventional homologous recombination techniques, and subsequently deleted the switch region and selectable markers by transient expression of cre[21]. Cells that had undergone cre-mediated deletion could be selected because of their concomitant loss of a herpes simplex virus thymidine kinase gene. An estimated 40–80% of ES cells expressing cre deleted the target fragment (2–4% of all cells), and these could give rise to transgenic mice. FLP recombinase has also been used in ES cells to delete a FRT-flanked selectable marker that would otherwise have interfered with experimental interpretation[20], but in this case there was no selection for the excision reaction and only a single excised clone was analysed. Excision occurred in 0.5% of all cells (approximately 10% of cells that took up DNA). Unfortunately, since different promoters were used in these experiments, it is not clear whether in ES cells, cre is more efficient than FLP[21].

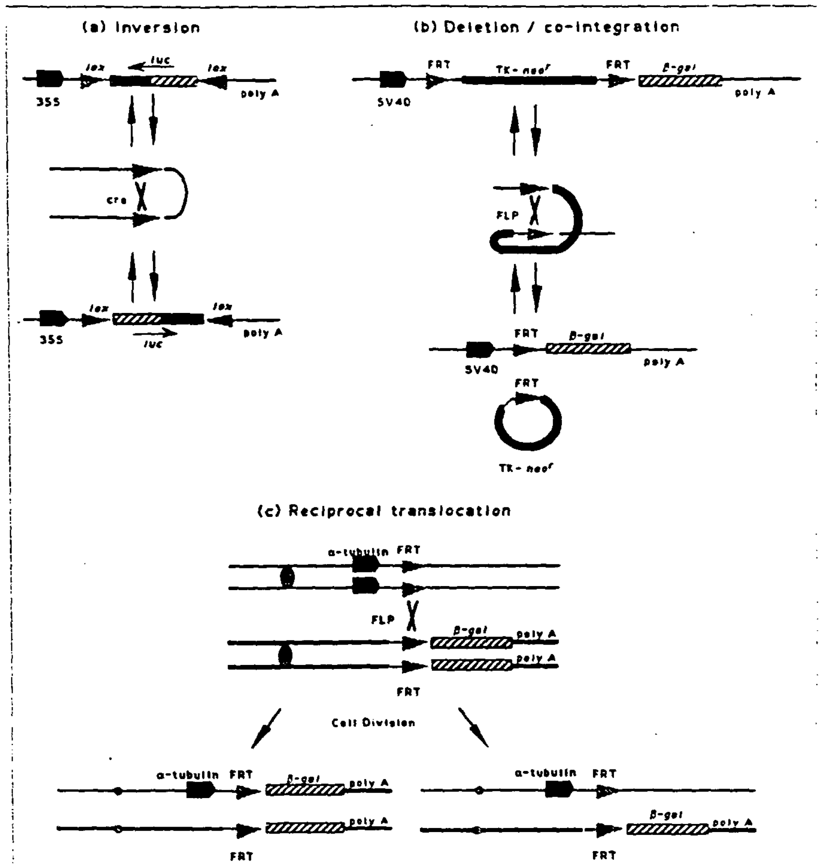

FIG 4

Examples of recombinase reactions *in vivo*. Supercoiled circles, relaxed and linear molecules are all substrates for the recombinases. Specific examples (redrawn from Refs 16, 17, 41, 43) show the types of rearrangements that can be produced. FRT, FLP recognition target; lox, cre recognition target; poly A, polyadenylation signal. (a) Site-specific recombination between two inverted target sites on a linear molecule inverts the intervening DNA[16]. Before recombination, the coding region of the luciferase (*luc*) gene was inverted with respect to a cauliflower mosaic virus 35S promoter. Cotransfection into tobacco protoplasts with a plasmid that expresses cre results in recombination and activation of *luc*. (b) Recombination between two sites in direct orientation leads to excision of the intervening DNA as a circular molecule that carries one copy of the target site. In this example from mammalian cells[17], expression of β-galactosidase (β-gal) from the SV40 early promoter is activated, with concomitant loss of G418 resistance (TK-*neo*^{r}). The second target remains on the linear molecule. The reverse of this reaction can also occur, leading to targeted integration of the circular molecule into the resident site on the linear DNA[17]. (c) If two linear molecules carry target sites, recombination leads to exchange of sequences distal to the recombination site, here activating expression of β-gal from an α-tubulin promoter[41]. This type of intermolecular recombination has been developed only in *Drosophila* between targets at identical positions on homologous chromosomes, and occurs after DNA replication: replicated sister chromatids are shown joined by their centromeres (shaded ovals). This event is FLP-mediated mitotic recombination[43]. Bottom: chromosomes of the two daughter cells after cell division, with their normal diploid DNA content.

Transgenics

Site-specific recombinases have applications in developmental biology in activating or removing genes at particular stages. Such controlled gene expression could mark a clone of cells for lineage studies, or allow the effect of lethal or deleterious sequences to be studied in a particular cell type or developmental stage. As a first step in this direction, transgenic mice expressing cre have been produced by microinjection. These were mated with mice carrying a lens-specific αA-crystallin promoter separated from a dormant SV40 T antigen coding sequence by an ingeniously designed stop sequence[34]. Since the stop sequence was flanked by directly repeated *lox* sites, cre-mediated excision led to activation of T antigen expression and the formation of lens tumours in every doubly transgenic animal. However, since transformation of a single cell would presumably be sufficient for tumorigenesis, the

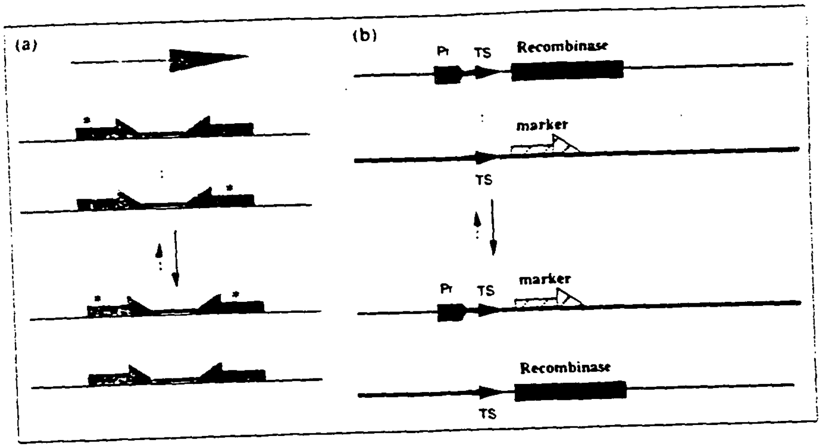

FIG 5

Strategies to reduce reversibility of the recombination reaction. (a) Binding-site mutations. During recombination, crossing-over occurs within the core region; this creates a new target, which consists of the left flank of one site joined to the right flank of the other. In the normal reaction, both sites are identical, so the new sites are also identical. However, if a mutation (*) is introduced into one binding site for each target, recombination will create a wild-type and a doubly mutant site. In principle, this allows the two directions of the reaction to be distinguished. A mutation of this type has been identified in FRTs (Fig. 3b); this reduces recombination efficiency only modestly when present in one FLP-binding site, but nearly abolishes recombination when present in both FRTs[48]. (Note that similar mutations can probably be obtained in the *loxP* target for cre.) *In vitro*, this conferred a tenfold advantage on the reaction in one direction[8], but when mutant sites were used *in vivo* they either were much less efficient than predicted[14] or reduced recovery of the desired recombination product[32]. (b) Inactivation of recombinase expression by promoter removal. If the recombinase is expressed so that its promoter (Pr) reads across its target site (TS), then recombination will separate the recombinase from its expression signals. The same promoter can drive a selectable marker gene that is switched on by the recombination event.

efficiency of cre recombination could not be assessed. Analogous experiments have used a strategy involving expression of a non-deleterious transgene to detect cre activity[35]. The cre coding region was driven by a thymus-specific promoter, and its expression led to loss of a β-galactosidase gene flanked by directly repeated *lox* sites. Although levels of β-galactosidase were too low to detect before these transgenics were crossed to cre mice, cre activity could be followed by loss of hybridization to β-galactosidase sequences. In some cases, the extensive array of transgenes was resolved down to a single insert.

cre-mediated recombination is also effective in transgenic tobacco and *Arabidopsis* plants[36–39]. Efficient loss (50–100%) of a marker gene flanked by *lox* sites was seen when plants that carried target sites were crossed to others expressing cre[36]. Deletion of unwanted transgene sequences followed by segregation and loss of the cre gene is valuable for removing bacterial marker genes from transgenic plants before field trials.

Perhaps the most elegant experiments involving site-specific recombinases have been conducted in *Drosophila*. Golic and Lindquist[10] constructed flies transgenic for a heat shock promoter–FLP construct and crossed these to flies whose sole copy of the cell-autonomous eye-colour gene *white* was flanked by directly repeated FRT sequences. Loss of *white*, causing white patches in the eye, was mediated by FLP expression both in somatic cells and in the germ line. The extent of mosaicism correlated with the severity and duration of the heat shock. By varying the timing of the heat shock, eye development could be visualized proceeding from posterior to anterior. FLP can also activate gene expression in any cell by mediating excision of a transcriptional stop sequence, and this activation is heritable. This approach is particularly useful for generating random clones of cells that are expressing genes at a specific time during development; for example, it has revealed that the wingless protein confers ventral positional information on cells and on their neighbouring wild-type tissue[40].

In *Drosophila*, FLP has also been exploited to enhance recombination between homologous chromosomes during mitosis, and thus provide an alternative method for marking a clone of cells and its descendants[11–13,41]. Although random mitotic recombination between homologous chromosomes can be induced by ionizing radiation, FLP provides a more efficient and specific method that has none of the lethal or mutagenic consequences associated with irradiation. Very high percentages of chimeric flies can be obtained and Xu and Rubin[42] have produced stocks of flies that have FRT sites on each major chromosome arm, providing a powerful system for clonal analysis and for rapid systematic screening of mutations in many biological processes (see Ref. 43). Activation of β-galactosidase by such a translocation between homologous chromosomes[41] is shown in Fig. 4c.

Chromosome engineering

Despite our ability to clone and map increasingly large segments of DNA, there is still a gulf between the limits of these analyses and the size of higher eukaryotic chromosomes. The fragility of very long DNA molecules makes it unlikely that *in vitro* approaches will be appropriate for handling multimegabase fragments in the foreseeable future. This has spurred research into *in vivo* DNA manipulation systems, including site-specific recombinases. Here, the great challenge remains the engineering of chromosomes by recombination between targets in non-homologous positions. If recombinases can act on distantly located sites, chromosomal rearrangements such as large deletions, inversions and translocations could be produced at will (Fig. 4).

Despite work in several laboratories, efficient long-distance recombination between non-homologous chromosomes in a higher eukaryote has yet to be

TABLE 1. Recombinase activity in cells and organisms

					Mammals			Plants			
Recombinase activities	***E. coli***	**Yeast**	***Drosophila***	**Mosquito embryos**	**Cultured cells**	**ES cells**	**Transgenic mice**	**Cultured cells**	**Transgenic tobacco**	**Transgenic *Arabidopsis***	**Refs**
Cre											
Deletion/inversion on plasmids	Yes	–	–	–	Yes[h]	–	–	Yes[c]	–	–	16,50
Deletion/inversion of integrated sequences	–	Yes	–	–	Yes	Yes	Yes	–	Yes	Yes	21,24,30,34–39,51
Co-integration of plasmids	Yes	–	–	–	–	–	–	Yes[c]	–	–	16
Targeted chromosomal integration	–	Yes	–	–	Yes[d]	–	–	–	–	–	15,22,33
Recombination of homologous chromosomes	–	–	–	–	–	–	–	–	–	–	
FLP											
Deletion/inversion on plasmids	Yes	Yes	–	Yes[c]	Yes[h]	–	–	Yes[f]	–	–	14,17,19,23
Deletion/inversion of integrated sequences	–	Yes[g]	Yes[a]	Yes	Yes[h]	Yes[h]	–	–	–	–	10,17,20,29,40
Co-integration of plasmids	Yes	Yes	Yes[a,i]	–	–	–	–	Yes[f]	–	–	14,19,23,31,45
Targeted chromosomal integration	Yes[j]	–	–	–	Yes[h]	–	–	Yes[f]	–	–	17,32
Recombination of homologous chromosomes	–	–	Yes[a]	–	–	–	–	–	–	–	11–13,41,42
R											
Deletion/inversion on plasmids	–	Yes	–	–	–	–	–	Yes[c]	–	–	8,18,47
Deletion/inversion of integrated sequences	–	Yes	–	–	–	–	–	Yes[c]	–	–	18
Co-integration of plasmids	–	Yes	–	–	–	–	–	–	–	–	8
Targeted chromosomal integration	–	–	–	–	–	–	–	–	–	–	
Recombination of homologous chromosomes	–	Yes	–	–	–	–	–	–	–	–	49

[a]Complete 599 bp 2μ repeat sequences used in all *Drosophila* experiments. [b]Also excision from herpes virus after infection. [c]In tobacco cells. [d]Also by co-injection or lipofection of purified cre protein. [e]Using complete 599 bp 2μ repeats and minimal 34 bp sequences. [f]Maize and rice protoplasts; using minimal 34 bp and wild-type 48 bp sites. [g]In *Pichia pastoris*. [h]Using wild-type 48 bp FRT sites. [i]By microinjection into the cytoplasm of *Drosophila* embryos. [j]Using minimal 34 bp FRT sites. – ; not done, to our knowledge.

reported. Tantalizingly, such experiments have been carried out in *S. cerevisiae*, using the pSR1 recombinase system[49]. Induction of the recombinase R gene resulted in efficient recombination, not only between directly repeated sites on the same chromosome to yield a 180 kb deletion (>80% efficiency), but also between sites on non-homologous chromosomes to produce a reciprocal exchange of chromosome arms (20% efficiency). However, the yeast genome is much smaller than that of mammals or plants, more tolerant of genomic rearrangement and lacks DNA modifications such as methylation and heterochromatin that could interfere with recombinase activity.

Nevertheless, these results in yeast hold the promise that systems for chromosome manipulation in higher eukaryotes may soon be developed, extending genetic engineering into the realms of 'genome engineering'. This would allow a more thorough understanding of the relationship between chromosomal position and gene expression, particularly in complex gene clusters, and a more precise investigation of imprinting and X-inactivation. Other developmental events that involve genomic rearrangement, such as immunoglobulin switching or activation of oncogenes by translocations, could be reconstructed with high efficiency in model systems, allowing more detailed investigation.

In plants, the tendency of transposable elements to jump to linked sites could be exploited to create deletions of various sizes. Recombinase target sites could be inserted into an appropriately engineered construct, such that one site is left behind and a second carried to an adjacent chromosomal location by a jumping transposable element. Crossing to recombinase-expressing plants would then cause deletion of the intervening DNA, and the progeny could be screened for any phenotype induced. The deleted region could be cloned readily from the original transgenic plant, since it would be flanked by the introduced construct and the transposon.

Conclusions

Site-specific recombinases can operate in heterologous environments with surprising efficiency, and promise to be powerful tools for the analysis of a number of difficult or intractable problems. Work published to date is largely concerned with establishing and testing recombinase systems. The next few years will no doubt see many more publications in which these approaches are used to tackle specific biological questions. They can already be used for '*in vivo* mutagenesis', bringing about activation or removal of specific genes; this application should prove particularly useful for studying potentially harmful mutations or deletions in adult organisms, since the genes involved can be maintained in a non-mutant configuration until exposed to recombinase activity by crossing or induction. Clonal and lineage analyses should benefit from a more efficient system of gene activation, whether using the 'enhanced mitotic recombination' developed for *Drosophila*[42] or the deletion/activation of cell markers when recombinase is activated in a particular tissue type or developmental stage.

Which recombinase is likely to prove the best? No firm conclusion can be drawn from the results discussed here. cre has been most widely used, and has been suggested to be more efficient in ES cells than FLP[21]; others have suggested that FLP and R, being of eukaryotic origin, may prove more efficient on chromatin-packaged DNA. For the foreseeable future, it is likely that work on exploiting all three systems will continue.

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N.J. Kilby, M.R. Snaith and J.A.H. Murray are in the Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge, UK CB2 1QT.

Yeast retrotransposons and tRNAs

DANIEL F. VOYTAS AND JEF D. BOEKE

The role of tRNAs in protein synthesis seems routine when compared with the novel ways in which the Ty retrotransposons of Saccharomyces cerevisiae use these interpreters of the genetic code. tRNAs and tRNA genes control essential steps in the retrotransposon life cycle by regulating protein expression, priming DNA synthesis and specifying integration target sites.

Transposable elements play an important role in shaping eukaryotic genomes. They affect chromosome structure by adding to the bulk of genomic DNA and provide regions of homology for ectopic recombination, which results in duplications, deletions and rearrangements. Transposable elements can also affect the structure and evolution of transcribed sequences through mutation, gene duplication and, in the case of mobile introns, intron loss or insertion.

However, the impact of transposition on evolution of the host genome is only half the story. The host, in turn, has a significant role in shaping the evolution of the transposable element. This is principally because the host provides many factors needed for transposable elements to replicate and proliferate. Because of the potentially disastrous consequences of transposition for the host and the role of host factors in transposition, the relationship between element and host necessarily involves coevolution of these two genetic systems.

Saccharomyces cerevisiae and its endogenous Ty transposable elements constitute a useful model for understanding interactions between element and host. Many of the host factors thus far described are fundamental to the cell and participate in vital cellular processes such as transcription[1], translation[2], RNA processing[3] and DNA repair[4,5]. Recent work has revealed that tRNA genes and tRNAs influence several key steps in transposition. Interactions between the Ty elements and *S. cerevisiae* tRNAs also reflect the intricate ways in which these transposable elements and their host have coevolved.

S. cerevisiae retrotransposons and the retrotransposon life cycle

Five distinct families of transposable elements, designated Ty1–Ty5, have been identified in *S. cerevisiae* (Fig. 1). Elements in all five families are retrotransposons; like the retroviruses, they are flanked by long terminal direct repeats (LTRs) and replicate by reverse transcription of a mRNA intermediate. The LTR retrotransposons fall into two distinct groups, referred to as the Ty1/copia and the Ty3/gypsy group retrotransposons after representative elements in *S. cerevisiae* and *Drosophila melanogaster*[6] (Fig. 1). Whereas Ty3 is the only member of the Ty3/gypsy group found in *S. cerevisiae*[7], there are four different families of Ty1/copia group elements. Ty1 and Ty2 are closely related and share extensive sequence similarity[8,9], Ty4 is a family of low-copy-number elements that is only distantly related to Ty1 and Ty2 (Refs 10, 11) and the one known member of the Ty5 family is a nonfunctional element near the left telomere of chromosome III (Ref. 12).

Transposition of both Ty1/copia and Ty3/gypsy group retrotransposons occurs by a mechanism that is strictly analogous to retroviral replication[13] (Fig. 2). The life cycle begins with transcription of an element resident in the host genome. The resultant mRNA is translated to yield protein products necessary for replication, and also serves as a template for the synthesis of new elements by reverse transcription. The two major polyproteins encoded by retrotransposons are equivalent to the products of the *gag* and *pol* genes of retroviruses. The products of the *gag* gene assemble into a virus-like core particle in the cytoplasm. Packaged inside this particle are the *pol* gene products, template mRNA, and a specific cellular tRNA that primes DNA synthesis by reverse transcription. Reverse transcription generates a DNA copy of the element, and this is integrated into the nuclear genome by integrase.

Studies of the yeast retrotransposon life cycle have been greatly facilitated by genetically marked Ty elements under inducible transcriptional controls[14,15].

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Site-specific Transgene Insertion: an Approach

Peter Wigley, Christiane Becker, Juliana Beltrame, Timothy Blake, Lesley Crocker, Sharon Harrison[A], Ian Lyons, Zara McKenzie[A], Rick Tearle[A], Robert Crawford[A] and Allan Robins[A]

Bresatec laboratories, Department of Biochemistry, University of Adelaide, S.A. 5005, Australia.
[A] *Bresatec Ltd, 39 Winwood St, Thebarton, S.A. 5031, Australia.*

Abstract. Methods to improve the production of transgenic animals are being developed. Conventional transgenesis, involving microinjection of DNA into fertilized eggs, has a number of limitations. These result from the inability to control both the site of transgene insertion and the number of gene copies inserted. The approach described seeks to overcome these problems and to allow single copy insertion of transgenes into a defined site in animal genomes. The method involves the use of embryonic stem cells, gene targeting and the FLP recombinase system.

Introduction

Animal transgenesis is a powerful technique for both basic and applied research (reviewed by Palmiter and Brinster 1986; Grosveld and Kollias 1992). Transgenic mice, rats, rabbits, sheep, cattle, goats and pigs have been produced by microinjection of linear DNA into fertilized eggs. In addition, transgenic mice have been generated from embryonic stem (ES) cells (Robertson 1987), usually in the context of gene 'knockout' experiments (Joyner 1993).

Transgenesis by means of microinjection has a number of limitations (Palmiter and Brinster 1986; Grosveld and Kollias 1992). The DNA integrates randomly into the genome, which may disrupt an endogenous gene or interfere with its expression. Random integration can also subject transgene expression to unwanted local influences (position effects), resulting from the proximity of regulatory elements (enhancers or silencers). In addition, transgenes often integrate in multiple copies at the one site, expression level is usually not proportional to transgene copy number, and multiple copies may result in increased transgene methylation (Mehtali *et al.* 1990) which can affect expression.

Some of the expression problems have been overcome by including dominant regulatory elements in the injected DNA such as the β-globin locus control region (LCR: Grosveld *et al.* 1987), or by including 'insulator' elements that shield the transgene from the influence of neighbouring regulatory elements (reviewed by Eissenberg and Elgin 1991). However, at this stage, there is no general means of ensuring copy number-dependent, position-independent expression of randomly-inserted transgenes.

We are developing technology to overcome these problems, allowing the site-specific, single-copy insertion of any gene into a mammalian genome without causing a deleterious mutation and without subjecting transgene expression to position effects. Our approach involves ES cells, gene targeting (homologous recombination) and the FLP recombinase system, each of which is summarized below.

ES Cells (reviewed by Robertson 1987)

ES cells were first isolated from mice about ten years ago. In recent years, they have become widely used particularly in gene knockout experiments (Joyner 1993). ES cells can be cultured in an undifferentiated state in the presence of the growth factor leukaemia-inhibiting factor (LIF). Genes can be inserted, disrupted and modified in the ES cell genome, and the altered cells can then be used to generate chimaeric animals. If the ES cells contribute to the germ line, the germ line chimaeras can be outcrossed to produce progeny that are heterozygous for the genomic modification. These animals can then be mated to produce offspring that are homozygous for the alteration.

ES cells are currently being developed for other species, e.g. rats, sheep, cows and pigs (A. French and M. Nottle, personal communication), and chimaeric pigs have recently been produced from ES cells (Wheeler 1994).

ES cell-like lines can also be generated from primordial germ cells (PGCs: reviewed by Rossant 1993), and chimaeric mice and chickens have been produced from PGC lines (Rossant 1993; Vick *et al.* 1993).

Gene Targeting (reviewed by Joyner 1993)

Gene targeting, or homologous recombination, was developed for use in mammalian cells in the mid to

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late 1980*s*. It has been extensively used in recent years for gene knockout experiments in ES cells. Animals carrying disrupted or modified genes are generated from the ES cells, allowing analysis of mutant phenotypes.

Briefly, homologous recombination involves generation of a targeting construct containing DNA homologous to the gene of interest, and a positive selection marker, e.g. the neomycin resistance gene. This construct is transfected into cells and, following selection, Southern analysis or polymerase chain reaction (PCR) analysis is used to distinguish between targeting events and random integrations. Various modifications have been developed to enrich for targeting events; in particular, the use of isogenic DNA to make the targeting construct has allowed targeting efficiencies up to 50% or better to be achieved in some cases.

FLP Recombinase (reviewed by Cox 1988)

FLP is a 45-kDa protein encoded by the 2-μ plasmid of the yeast *Saccharomyces cerevisiae*. FLP acts as a site-specific recombinase by binding to the FLP recombination target (FRT) site. The minimal functional FRT site is approximately 30 bp consisting of two 13-bp repeats inverted with respect to each other and separated by an 8-bp spacer. A monomer of FLP binds to each repeat and the DNA is cut at each end of the spacer region. FLP can catalyse both DNA excision (intramolecular) and insertion (intermolecular). FLP has been shown to work *in vitro* and in a variety of systems including mammalian cells.

In cultured mammalian cells, by means of a colour selection assay involving the *lacZ* gene, O'Gorman *et al.* (1991) demonstrated FLP-mediated excision and insertion reactions at genomic FRT sites. Excision has also been shown to occur in mouse ES cells (Jung *et al.* 1993).

The major problem with the FLP system is the inability to control the direction of recombination. Excision and insertion are reversible reactions and excision is highly favoured over insertion since it is intramolecular as opposed to intermolecular. However, experiments with a model system in bacteria have shown that insertion events can be stabilized by the delivery of a pulse of FLP activity (Huang *et al.* 1991).

Approach

Our approach for precise transgenesis involves the following steps:

(1) gene targeting to introduce an FRT site into a specific genomic locus in ES cells, thereby creating the 'transgene acceptor site';

(2) single-copy insertion of transgenes into the targeted FRT site using FLP recombinase; and

(3) generation of transgenic animals from the modified ES cells.

Genomic Insertion Locus

The genomic insertion site will be a histone H4 gene locus. This choice fulfils the two major criteria for an insertion site described below.

(1) Insertion will not cause a deleterious mutation. Histones are small basic proteins that package DNA into nucleosomes in all eukaryotic cells (Stein *et al.* 1984). There are five major classes of histone: H1, H2A, H2B, H3 and H4. Histone genes are repeated approximately 10–20 times in mammalian genomes (Stein *et al.* 1984) and, in the case of H4, each gene is predicted to encode an identical protein (Stein *et al.* 1984; Wells and Brown 1991). Therefore, disruption of one H4 gene should have no phenotypic consequences when transgenic animals are bred to homozygosity.

(2) Position effects on transgene expression will be minimized or eliminated. Histone genes are expressed in all cell types (Stein *et al.* 1984). Therefore, we predict that the H4 gene locus will be 'available' for expression in all cell types, i.e. access to transcription machinery will be possible in all cells. Thus, position effects will be minimized or eliminated and the level and pattern of transgene expression should depend only on the regulatory elements included with the transgene.

Creating the Transgene Acceptor Site (TAS)

Generating the TAS will involve the two steps described below.

(1) Gene targeting will be used to insert a 'FRT–neo–FRT' cassette into one histone H4 gene locus in ES cells (described in detail below). This cassette will contain a fully-functional neomycin resistance gene (*neo* gene) flanked by direct repeats of the FRT site. The *neo* gene will act as a positive selection marker and targeting events will be detected by Southern analysis.

(2) ES cells with the FRT–neo–FRT cassette integrated into the H4 locus will be transfected with a source of FLP recombinase in order to excise the *neo* gene. This will generate a single FRT site in the H4 locus, i.e. the TAS.

Gene Insertion into the TAS

FLP recombinase will be used to insert genes, in a single copy, into the TAS. This will involve transfection of the TAS ES cell line with a source of FLP plus a plasmid containing the gene of interest (with regulatory elements) linked to a single FRT. Insertion events will be characterized by Southern analysis. The modified ES cells will then be used to generate transgenic animals.

Progress

Model System

We are developing the transgene insertion technology with the mouse as a model system since ES cells are available for this animal. We are also attempting to isolate porcine ES cells. The insertion technology will be adapted to the pig system once the authenticity of our putative pig ES cells is confirmed by the generation of germ line chimaeras.

Targeting of FRT–neo–FRT into a Mouse H4 Gene

We have made a genomic DNA library from E14 ES cells (Hooper *et al.* 1987) and by means of a chicken histone H4 gene probe (Wang *et al.* 1985), we have isolated genomic clones containing mouse H4 genes. A targeting construct has been made which contains the FRT–neo–FRT cassette and 8 kb of genomic DNA from one H4 locus. This DNA is isogenic with the genome of the ES cells, ensuring efficient targeting (Grosveld and Kollias 1993). Targeting experiments are currently in progress.

Establishing and Optimizing the FLP Recombinase System in Mouse ES Cells

We have adapted an FLP recombinase assay system developed by O'Gorman *et al.* (1991). Excision and insertion reactions are first identified by a colour detection assay utilizing the gene for β-galactosidase, and subsequently confirmed by means of Southern analysis.

We are taking the following two approaches to supplying a pulse of FLP activity to ES cells:

(1) transfection of the FLP gene under the control of the interferon-inducible human '6–16' promoter (Porter *et al.* 1988; Whyatt *et al.* 1993); and

(2) transfection of bacterially-produced FLP protein (from the PS731 strain) (Pan *et al.* 1991).

Both approaches have been used successfully to demonstrate FLP-mediated excision. Insertion experiments are under way.

Alternative Approaches

Gene Insertion by means of Homologous Recombination

As an alternative to FLP-mediated gene insertion, we will also attempt to insert genes by homologous recombination in ES cells. Constructs will be made for targeting into the H4 gene locus which will contain the gene to be inserted in addition to the FRT–neo–FRT cassette. Following targeting, the *neo* gene will be excised by means of FLP. Therefore, only the inserted transgene and a single FRT will remain in the genome. The modified ES cells will then be used to generate transgenic animals.

FLP in Fertilized Eggs

We will also attempt FLP-mediated gene insertion in fertilized eggs from animals transgenic for the FRT site. These animals will be generated in the following two ways:

(1) microinjection of FRT-containing DNA into fertilized eggs; and

(2) generation of animals from ES cells carrying a targeted genomic FRT site.

In the former case, the FRT sites will be randomly integrated. However, it may be possible to select an integration event which: *(i)* does not cause a mutation; and *(ii)* provides a site that does not interfere with expression of inserted genes.

Once FRT-transgenic animals are generated, we will attempt to carry out FLP-mediated insertion by injection of FLP protein, together with a plasmid containing the gene of interest linked to a single FRT, directly into fertilized eggs. Ultimately, this may allow us to insert genes into a defined site in the genome without using ES cells.

Acknowledgments

The original basis of this work was an idea of Dr Julian Wells who died in 1993. The authors would like to acknowledge the many outstanding contributions made by Julian during his distinguished career, particularly in the areas of gene regulation and animal transgenesis. Our work is supported by the Transgenic R & D Syndicate No. 1.

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Genetics

Targeted oncogene activation by site-specific recombination in transgenic mice

(*cre/lox*/lens development/simian virus 40 large tumor antigen)

M. LAKSO*†, B. SAUER‡, B. MOSINGER, JR.*, E. J. LEE*, R. W. MANNING‡, S.-H. YU*, K. L. MULDER*, AND H. WESTPHAL*

*Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892; and ‡Du Pont-Merck Pharmaceuticals, Inc., Experimental Station E328, Wilmington, DE 19880-0328

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ABSTRACT An efficient and accurate method for controlled *in vivo* transgene modulation by site-directed recombination is described. Seven transgenic mouse founder lines were produced carrying the murine lens-specific αA-crystallin promoter and the simian virus 40 large tumor-antigen gene sequence, separated by a 1.3-kilobase-pair Stop sequence that contains elements preventing expression of the large tumor-antigen gene and Cre recombinase recognition sites. Progeny from two of these lines were mated with transgenic mice expressing the Cre recombinase under control of either the murine αA-crystallin promoter or the human cytomegalovirus promoter. All double-transgenic offspring developed lens tumors. Subsequent analysis confirmed that tumor formation resulted from large tumor-antigen activation via site-specific, Cre-mediated deletion of Stop sequences.

A desired goal of transgene technology is efficient and accurate manipulation of DNA sequences after their integration in the germ line. DNA recombinases that mediate integration or excision of sequences at specific recognition sites in both prokaryotic (1–5) and eukaryotic (6–10) systems are well suited for this purpose. The bacteriophage P1 recombinase Cre catalyzes reciprocal recombination at a specific locus of crossing over (*lox*) (11–16). The *lox* sequence is c mposed of two 13-base-pair (bp) inverted repeats separated by an 8-bp spacer region. Upon binding to the inverted repeats, Cre synapses with a second *lox* site and then cleaves the DNA in the spacer region to initiate strand exchange with the synapsed *lox* partner. No additional factors are required in the recombination.

In this study, we examine the potential of the *cre/lox* system to activate a dormant transgene in the mouse. The simian virus 40 (SV40) large tumor antigens (TAgs) directed to the lens by a murine αA-crystallin promoter (mαA) cause malignant lens tumors (17). We inserted between mαA and TAg a specially designed Stop sequence that prevents gene expression and is flanked by *lox* sequences. By crossing the dormant TAg transgenic mouse lines with Cre-expressing transgenic lines, we report here that the Cre protein recognizes the *lox* sites of the mαA–Stop–TAg transgene and recombines the two *lox* sequences, thereby removing Stop and activating TAg. Our studies show that targeted transgene modification in the mouse can be performed efficiently and accurately with a prokaryotic recombinase.

MATERIALS AND METHODS

Recombinant DNA Constructs and Transgenic Mice. The mαA–Stop–TAg transgene was constructed from the pr viously described plasmid pαA366a-T (17) by inserting to the *Bam*HI site between mαA and TAg a 1.3-kbp Stop fragment flanked by directly repeated *lox* sequences (5′-ATAACTTCGTATAGCATACATTATACGAAGTTAT-3′) (Fig. 1). The Stop sequence was composed of the 550-bp C-terminal sequence of yeast *His3* gene, 825 bp of the SV40 polyadenylylation signal region, and a synthetic oligonucleotide (5′-GATCTGACAATGGTAAGTAAGCTT-3′, where ATG is a false translation initiation signal and GTAAGT is a 5′ splice donor site). The human cytomegalovirus (hCMV)–*cre* construct was obtained by fusing the 1.2-kbp *cre* gene to the hCMV promoter as described (16) and the mαA–*cre* construct was derived from the hCMV–*cre* construct by changing the promoter sequence and by a T to G substitution at the −3 position. Transgenic mice were produced as described (18).

Screening of Transgenic Mice. The genotypes of all offspring were analyzed both by PCR and by Southern blots. For PCR analysis, mouse tail DNAs (2 μg) were amplified by 35 cycles (1 min 20 sec, 92°C; 1 min, 65°C; 1 min 30 sec, 72°C) on a thermal cycler. The 5′ primer for *cre* was 5′-GGACATGTTCAGGGATCGCCAGGCG-3′ and the 3′ primer was 5′-GCATAACCAGTGAAACAGCATTGCTG-3′. The 5′ and 3′ primers for TAg were 5′-GGTCTTGAAAGGAGTGCCCTGGGGGA-3′ and 5′-CCTCAGTTGCATCCCAGAAGCCTCC-3′, respectively. Twenty percent of the reaction volume was analyzed on a 1.75% agarose gel. For Southern blot analysis, tail genomic DNAs (5 μg) were digested with *Bam*HI and separated on a 0.75% agarose gel. Transfer to GeneScreen filters (DuPont) and Southern hybridization were performed according to Maniatis *et al.* (19). A 2.2-kbp *Taq* I/*Bam*HI fragment of SV40 viral DNA (Bethesda Research Laboratories) and a 0.4-kbp *Bam*HI fragment of the 5′ portion of *cre* were used as hybridization probes. The expression levels of the *cre* transgenes were determined either by a functional test in fibroblasts and kidney cells derived from hCMV–*cre* transgenic mice (20) or by a PCR amplification of cDNAs synthesized from total RNAs from mαA–*cre* lenses. Lens RNAs (2 μg) were converted to single-strand cDNA by Moloney murine leukemia virus reverse transcriptase and were amplified by 35 cycles of PCR as described above.

Phenotype Analysis of Mouse Lenses. Mouse eyes were fixed in 4% paraformaldehyde or 4% glutaraldehyde and 36.8% formaldehyde, embedded in methacrylate, sectioned, and stained with hematoxylin and eosin as described (21). Immunoperoxidase staining with an antibody specific to TAg was performed on frozen tissue sections (17) under the conditions previously reported (22), except that nonspecific

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Abbreviations: SV40, simian virus 40; TAg, large tumor antigen; mαA, murine αA-crystallin promoter; hCMV, human cytomegalovirus promoter/enhancer.
†To whom reprint requests should be addressed.

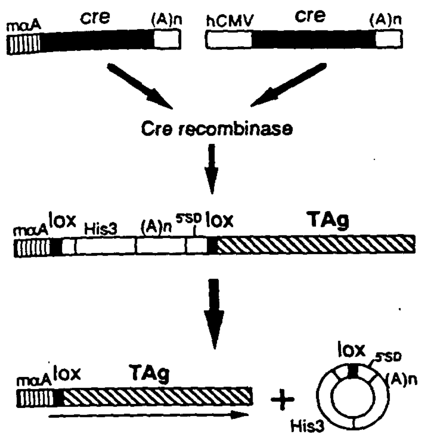

FIG. 1. DNA constructs used for generating transgenic mice. The *Nci* I fragment microinjected into fertilized mouse eggs is shown. mαA–Stop–TAg contains the mαA promoter separated from the SV40 TAg sequences by the 1.3-kbp regulatory Stop fragment, which was flanked by directly repeated *lox* sites and inserted into the *Bam*HI site between the mαA promoter and the TAg gene of the previously described plasmid pαA366a-T (17). The 3.3-kbp *Hin*dIII fragment of pBS187 plasmid (16) contained the *cre* gene under the control of the hCMV promoter/enhancer (hCMV–*cre*). The 3.0-kbp *Sma* I/*Hin*dIII fragment containing mαA promoter and *cre* gene (mαA–*cre*) was derived from pBS187 by changing the promoter sequence and by T to G substitution at the −3 position for translation enhancement (16). (A)n, polyadenylylation signal; 5'SD, 5' splice donor site.

binding was blocked with normal goat serum from the Vectastain detection kit (Vector Laboratories).

Molecular Analysis of Double-Transgenic Mouse Genotypes. Total genomic DNAs (1 μg) of eyes and/or tails from each mouse were amplified with a 5' primer (5'-GCTCCTGTCT-GACTCACTGCCAG-3') identical to the mαA promoter sequence and a 3' primer (5'-CCCCCAGGCACTCCTTTCAA-GACC-3') complementary to the TAg first exon sequence. Amplification occurred in 100 μl of PCR buffer with 1.6 mg of bovine serum albumin per ml by 35 cycles of PCR (1 min 20 sec, 96°C; 3 min 30 sec, 75°C with 5-sec automatic cycle extension). One-third volume of the PCR amplification products was loaded on 1.75% agarose gel for analysis. The 220-bp PCR amplification products were then isolated from the agarose gel by electroelution and cloned into pGEM4Z plasmid (Promega). Double-stranded DNAs were sequenced as described (23).

RESULTS AND DISCUSSION

The *cre*/*lox* Binary System. The design of our experiment calls for accumulation of chromosomal *lox* sites and active Cre recombinase in specific target cells of transgenic mice. In this constellation, the recombinase would excise a Stop signal that prevents expression of SV40 tumor antigens and would thereby initiate oncogenesis in the target tissue. The removal of even a single Stop sequence at an integration site that may contain more than one transgene copy would activate the oncogene.

Production of Transgenic Mice Carrying Either the Dormant TAg Oncogene or the Cre Recombinase Gene. The mαA–Stop–TAg dormant oncogene construct was designed to contain the 1.3-kbp Stop fragment inserted between mαA and TAg (Fig. 1). The Stop sequence contained a 550-bp spacer derived from C-terminal sequences of the yeast *HIS3* gene to enhance the efficiency of the downstream 825-bp SV40 polyadenylylation signal in terminating transcripts initiated by the mαA pr moter. An additional safeguard to prevent TAg expression was a synthetic oligonucleotide, including a false translation initiation signal (ATG), and a 5' splice donor site (GTAAGT). The Stop fragment was flanked by directly repeated *lox* sequences. Cre expression constructs were obtained by fusing the 1.2-kbp *cre* gene to the mαA promoter (mαA–*cre*) or to the hCMV promoter (hCMV–*cre*). In the mαA–*cre* construct, a T to G nucleotide substitution was introduced to the −3 position relative to the AUG codon to enhance translation (16).

Transgenic mice were produced by injecting separately all three gene constructs as linear fragments into the pronuclei of fertilized mouse eggs that were then transferred to oviducts of pseudopregnant foster mothers (18). Seven independent transgenic mouse founder lines harboring the mαA–Stop–TAg construct exhibited normal translucent lenses, indicating that the Stop sequences effectively prevented TAg expression. Seven founder animals with the mαA–*cre* transgene and three founders with the hCMV–*cre* transgene were also obtained. No abnormalities among founders or their offspring were observed. High *cre* expressor lines were selected based (*i*) on the presence of functional Cre enzym in fibroblasts and kidney cells from hCMV–*cre* transgenic lines or (*ii*) on a PCR analysis of cDNAs synthesized from total RNAs of mαA–*cre* lenses as described in *Materials and Methods*. F_1 progeny of two mαA–Stop–TAg founder lines, mαA–Stop–TAg1 with >50 copies and mαA–Stop–TAg2 with ≈50 copies of the transgene per haploid genome, were mated with those mαA–*cre* and hCMV–*cre* transgenic lines that showed high *cre* expression (Table 1).

Table 1. Transgenic mouse lines and occurrence of tumors

Genotype	No. of animals	No. of animals with lens tumors
	Experiment 1*	
mαA–Stop–TAg1	70	0
mαA–Stop–TAg2	21	0
mαA–*cre*	>100	0
hCMV–*cre*	>100	0
mαA–Stop–TAg1/mαA–*cre*	17	17
mαA–Stop–TAg1/hCMV–*cre*	10	10
mαA–Stop–TAg2/mαA–*cre*	4	4
mαA–Stop–TAg2/hCMV–*cre*	5	5
	Experiment 2†	
mαA–Stop–TAg1/mαA–*cre*	10	10
mαA–Stop–TAg1	9	0
mαA–*cre*	11	0
WT	10	0
	Experiment 3‡	
mαA–Stop–TAg1/mαA–*cre*	16	16
mαA–Stop–TAg1	7	0
mαA–*cre*	8	0
WT	1	0

*The genotypes of the animals were determined by PCR and Southern blot analysis. From the seven transgenic mouse founder lines carrying the dormant mαA–Stop–TAg gene, two were selected for mating. The A9874 founder line of the mαA–*cre* lines was selected for its high *cre* expression based on lens RNA analysis by PCR. The hCMV–*cre* founder line DP769 was also chosen because of its high *cre* expression based on Cre activity as described. All 36 double-transgenic animals had cataracts and all single transgenics had normal eyes.

†Analysis of four successive litters of F_1 generation single transgenic mαA–*cre* female and F_1 generation single-transgenic mαA–Stop–TAg1 male. Genotypes of all offspring (n = 40) were confirmed by PCR and Southern analysis. WT, wild type.

‡Analysis of offspring from a cross between mαA–St p–TAg1/mαA–*cre* double-transgenic female and male (produced by matings as described in †), which were heterozygous for each transgene. Genotypes of all offspring (n = 32) were analyzed by PCR. WT, wild type.

All Double-Transgenic Offspring Exhibit Lens Cataracts. The double-transgenic offspring harboring both the mαA-Stop-TAg and mαA-*cre* or hCMV-*cre* transgenes were easily identified on the basis of lens cataracts upon eye opening 10 days after birth (Fig. 2*B*). The genotypes of all offspring were confirmed by PCR (Fig. 3*A*) and Southern blot (Fig. 3*C*) analysis of DNA obtained from tail biopsies at weaning age. Two separate matings between mαA-Stop-TAg1 and mαA-*cre* progeny generated 17 double transgenics, each with cataracts in both lenses, from a total of 65 pups. Likewise, double transgenics resulting from crosses between mαA-Stop-TAg2 and mαA-*cre* or between mαA-Stop-TAg1 or -2 and hCMV-*cre* all had cataractous lenses at expected Mendelian frequencies (Table 1). More recently, crossing both *cre* transgenic lines with a third mαA-Stop-TAg transgenic founder line with fewer copies than the first two founder lines has also resulted in offspring that exhibited cataractous eyes at expected Mendelian frequencies (data not shown).

Malignant Transformation Results from TAg Expression. Histological analysis of lens sections obtained from double-transgenic animals consistently revealed morphological changes characteristic of proliferating lens tumors (Fig. 2*C*). Instead of the single mitotically active layer of anterior epithelial cells seen in normal lenses of mαA-Stop-TAg transgenic mice (data not shown), a multilayered heterogenous epithelium consisting of anaplastic cells was observed in the anterior part of the double-transgenic lenses. Tongues of invasive growth were extending into the rest of the disorganized lens mass. The lens mass was punctuated with large cysts that most likely result from improper elongation of fiber cells. Immunostaining of frozen lens sections with a polyclonal antibody to TAg showed a strong signal in the anaplastic cell mass (Fig. 2*D*). TAg could not be detected in lenses of mαA-Stop-TAg littermates (data not shown). Taken together, thes results strongly suggest that the malignant transformation was du to TAg expression in the lenses of double-transgenic animals.

TAg Activation Is Due to Site-Specific Recombination in the Embryonic Mouse Genome. The activation of the dormant TAg by Cre was assessed by PCR analysis of transgene sequences in affected and control lenses. A 24-bp 3′ primer complementary to the first exon sequence of TAg and a 23-bp 5′ primer identical to the mαA promoter sequence were synthesized. Amplifications with these primers predictably generate a 220-bp fragment from mαA-TAg, representing th product of Cre-mediated Stop excision at the mαA-Stop-TAg locus. The expected 220-bp fragment was indeed obtained with genomic DNA from double-transgenic mice lenses, but not with genomic DNA from control αA-Stop-TAg mouse lenses (Fig. 4*A*). Sequence analysis of amplified 220-bp cDNA fragments revealed one single *lox* site flank d by 5′ mαA promoter sequences and 3′ TAg (Fig. 4*B*). This confirms that TAg activation had occurred, as predicted, via precise site-specific deletion of the Stop sequences in the genome of differentiating lens cells. While Stop excision

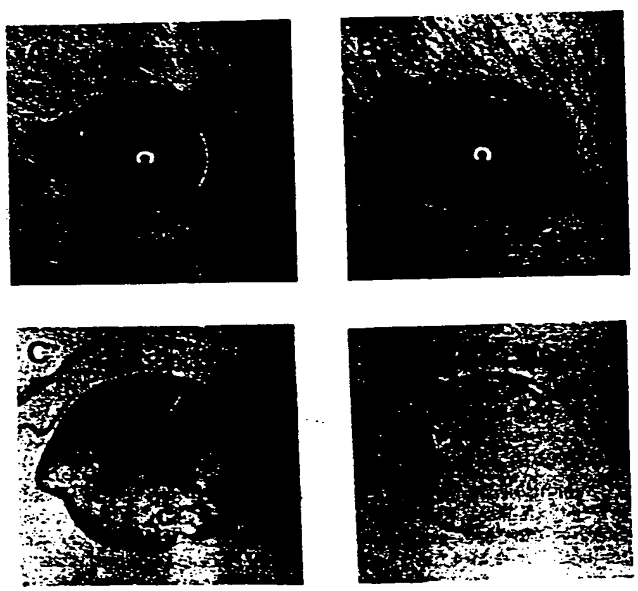

FIG. 2. Phenotype of double-transgenic mice bearing both mαA-Stop-TAg and mαA-*cre* genes. (*A*) Eye of a normal mαA-Stop-TAg1 single-transgenic mouse at 10 weeks of age. The albino mouse eye appears red. (*B*) Eye of a mαA-Stop-TAg1/mαA-*cre* double-transgenic sibling. (*C*) Lens pathology of a double-transgenic mouse. Hematoxylin and eosin-stained eye section from a 16-day-old mαA-Stop-TAg1/mαA-*cre* mouse. Aqueous humor (a), vitreous humor (v), cyst (c), and anaplastic cells (arrowhead) are shown. (×8.) (*D*) Indirect immunostaining with an antibody specific to TAg (22) of a lens section from the same animal as in *C*. Anterior epithelial cells (arrowhead) are strongly stained. The antibody did not stain lenses from single-transgenic littermates. (×6.)

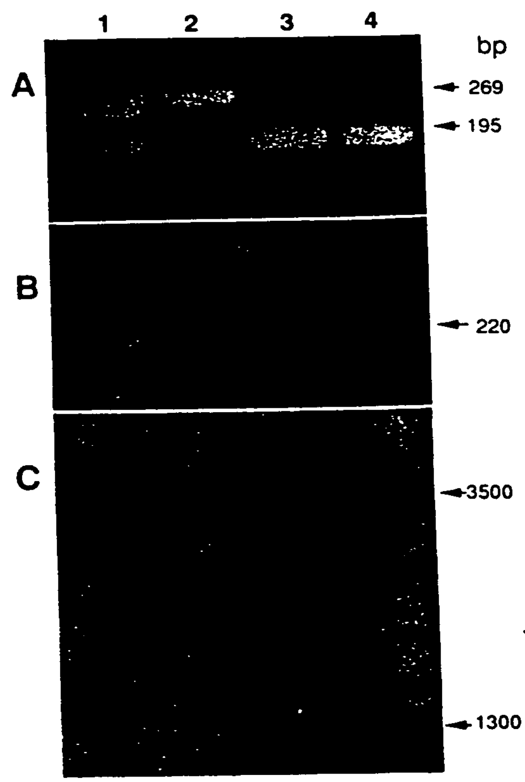

FIG. 3. Genotypes of a mαA-Stop-TAg1 × hCMV-*cre* litter. (*A*) Identification of mαA-Stop-TAg and hCMV-*cre* transgenes by PCR amplification of tail DNA. Primers for TAg generated a 195-bp fragment and primers for *cre* generated a 269-bp fragment. PCR products were separated on a 1.75% agarose gel for analysis. Lanes: 1, offspring 1; 2, offspring 2; 3, offspring 3; 4, offspring 4. Offspring 1, 3, and 4 are double transgenic. (*B*) Identification of the Stop deletion from tail genomic DNAs from the same animals. The PCR strategy selected is shown in Fig. 4*A*. Amplificati n yielded the expected 220-bp fragment from the three double-transgenic offspring 1, 3, and 4. (*C*) Southern hybridization analysis of *Bam*HI-digested tail DNAs from the same animals. The band of offspring 1 represents a single-copy *Bam*HI fragment of the TAg, indicating that only one mαA-TAg transgene is left in the genome, and Stop sequences (1.3 kbp) have been excised. Offspring 3 and 4 retain multiple copies of mαA-Stop-TAg transgenes in their genomes.

Attachment E

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Genetics

Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice

SUSAN M. DYMECKI

Department of Embryology, Carnegie Institution of Washington, Baltimore, MD 21210

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ABSTRACT Site-specific recombinases are being developed as tools for "*in vivo*" genetic engineering because they can catalyze precise excisions, integrations, inversions, or translocations of DNA between their distinct recognition target sites. Here it is demonstrated that Flp recombinase can effectively mediate site-specific excisional recombination in mouse embryonic stem cells, in differentiating embryonal carcinoma cells, and in transgenic mice. Broad Flp expression is compatible with normal development, suggesting that Flp can be used to catalyze recombination in most cell types. These properties indicate that Flp can be exploited to make prescribed alterations in the mouse genome.

Site-specific recombinases are being developed as tools for genetic engineering because of their simplicity and precise activity in a variety of organisms. Two well-studied recombinases include Flp, from *Saccharomyces cerevisiae*, and Cre, from bacteriophage P1: both have been shown to catalyze excisions, integrations, inversions, or translocations of DNA between their distinct recognition target sites without requiring added cofactors (1–6). The type of recombination reaction is determined by the orientation of target sites relative to each other on a segment of DNA: in particular, directly repeated sites specify excision of intervening DNA.

Controlled recombinase expression in an organism carrying appropriately placed target sites can be exploited to alter the genotype of subsets of cells within an otherwise normal embryo or adult. Such mosaic animals bearing clones of genetically distinct somatic cells have been most extensively generated in *Drosophila* using Flp, providing the means to address previously intractable problems. For example, Flp-mediated excisional recombination has been used to irreversibly activate a marker gene in specific cell populations and their descendants, allowing cell lineages to be studied (7, 8); similarly, genes have been ectopically expressed to study their effects on pattern formation (9). By promoting mitotic exchange between target sites on homologous *Drosophila* chromosomes, Flp has provided an effective methodology for F_1 genetic screens (10–12). In mammalian cell culture, Flp has been shown to effectively catalyze both excision and integration of DNA at specific chromosomal sites (13–16). By catalyzing recombination between target sites on the same DNA molecule or by promoting translocations between targets sites on different DNA molecules, site-specific recombinases can be used to study a variety of biological processes. Importantly, such recombination schemes can be used to generate tissue- or stage-specific mutations that would be lethal if generated in the whole organism.

To establish some of these methods in the mouse, it may require using both homologous (gene replacement)- and site-specific recombination in embryonic stem (ES) cells to precisely place target sites in the genome. Consequently, the properties of a given recombinase should be delineated in both ES cell culture and the mouse. While Cre-mediated recombination has been successfully employed (17–21), the utility of Flp recombinase in ES cells and the mouse has not been established. Developing the technology to engineer multiple recombination reactions (independent gene activation or deletion events) using both Flp and Cre should significantly augment the tools available for molecular studies in mice. Here the utility of Flp to excise DNA in ES cells, differentiating embryonal carcinoma (EC) cells, and in transgenic mice is investigated.

MATERIALS AND METHODS

Plasmid Constructions and Production of Transgenic Mice. The *lacZ* target vector containing Flp recombinase target (FRT) sites (pFRTZ: Fig. 1*A*) was generated by inserting the *Hin*dIII/*Sal*I fragment from pSLhβAPr-lacZ-pA (22) containing human β-actin gene (h*ACTB*) sequences [3-kb 5′ flank, 78-bp 5′ untranslated region, and 832-bp first intron; ref. 23] into the unique *Hin*dIII and *Sal*I sites of pFRT$_2$neo.lacZ (24). The control plasmid pFRTZ-product was constructed by inserting the same h*ACTB* *Hin*dIII/*Sal*I fragment into pFRT.-lacZ (24). A variant of pFRTZ (designated pFRTZ.2) was generated by inserting the 1.9-kb *Xho*I/*Sal*I fragment from pIC19R-MC1TK (25) containing the herpes simplex virus thymidine kinase (HSV-tk) gene between the FRT sequences of pFRTZ. The prototype plasmid pNEOβ-GAL (ref. 13: Stratagene) was also used as target DNA. The *FLP* transgene expression vector, phACTB::FLP (Fig. 1*B*), was constructed by inserting the 3.9-kb *Xba*I/*Sal*I fragment from pSLhβAPr-lacZ-pA into the unique *Xba*I site of pFLP (24). A nonexpressing, negative control *FLP* vector (pRevhACTB::FLP) was constructed, which contains identical h*ACTB* sequences in reverse orientation. To generate pWnt1::FLP, the 2-kb *Sal*I fragment from pFLP, containing a synthetic intron, the sequence encoding Flp (ref. 13: Stratagene), and simian virus 40 early polyadenylylation (pA) sequence, was inserted into the unique *Eco*RV site of pWEXP2 (26). To produce transgenic mice, transgenes were purified away from plasmid sequences and injected into fertilized eggs from B6SJLF$_1$ × B6SJLF$_1$ mice as described (27).

Cell Culture. CCE ES cells (28) were plated onto mitomycin C-treated STO fibroblasts (29) in DMEM supplemented with 15% fetal bovine serum (FBS), 2 mM glutamine, 0.1 mM 2-mercaptoethanol, 2000 units/ml of leukemia inhibitory factor (ESGRO, GIBCO/BRL), 0.1 mM MEM nonessential amino acids, 30 μM nucleosides. Primary embryonic fibroblasts (EF) were prepared from hemizygous transgenic embryos 13.5 days post coitum as described (29). P19 EC cells

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Abbreviations: FRT, Flp recombination target; *FRTZ*, FRT-disrupted *lacZ* transgene; h*ACTB*; human β-actin gene; RA, retinoic acid; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; β-Gal, β-galactosidase; ES, embryonic stem; EC, embryonal carcinoma; EF, embryonic fibroblast.

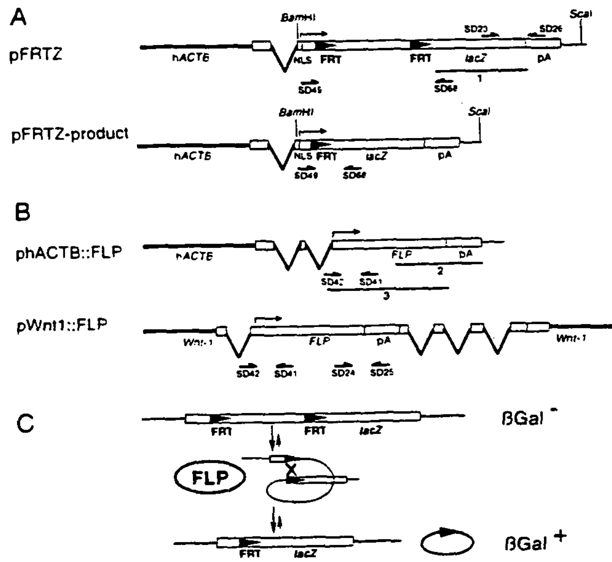

FIG. 1. DNA constructs and the Flp-mediated recombination event. (*A* and *B*) Structure of target and recombinase transgenes. FRTs are depicted as black triangles. Rectangles represent exons; heavy lines, introns and flanking regulatory sequences; thin lines, vector sequences; arrows, translation start sites. Hybridization probes are represented by numbered lines. PCR oligonucleotide primers are represented by half arrows. (*A*) Structure of target transgenes. Plasmid pFRTZ (for FRT-disrupted *lacZ* transgene) contains 3.9 kb of sequence from the human β-actin (h*ACTB*) gene (22, 23) inserted into the target vector pFRT$_2$neo.lacZ (24); a nuclear localization signal (NLS) and simian virus 40 early polyadenylation (pA) sequence are also included. Although not shown, pFRTZ.2 is an alternative target plasmid that contains the HSV-tk gene inserted between the FRT sequences of pFRTZ and is relevant to transfections shown in Fig. 3. Control plasmid pFRTZ-product represents the product of Flp-mediated excisional recombination. Restriction sites and probe 1 used in the Southern blot analysis of Fig. 4*B* are shown on pFRTZ. (*B*) Structure of *FLP* transgenes. Plasmid phACTB::FLP contains the 3.9-kb h*ACTB* fragment inserted into the expression vector pFLP (24), which contains a synthetic intron, Flp-encoding sequence, and simian virus 40 late pA sequence from pOG44 (ref. 13; Stratagene). Although not diagrammed, pRevhACTB::FLP contains the h*ACTB* sequences in reverse orientation and serves as a negative control. Plasmid pWnt1::FLP contains the synthetic intron, Flp-encoding sequence, and the simian virus 40 late pA from pFLP inserted into the polylinker of the *Wnt-1* expression vector pWEXP2 (26). Probe 2 is relevant to the whole mount *in situ* hybridization analyses shown in Fig. 3; probe 3 is used in Northern blot analyses of Fig. 3. (*C*) Diagram of the *FLP*-mediated excisional recombination reaction.

were maintained in a 1:1 mixture of DMEM and Ham F2 medium supplemented with 7.5% FBS/2 mM glutamine.

Transient Transfections. Transient transfection of ES cells (2×10^5 ES cells in 3.5-cm dishes) was by lipofection (Lipofectamine, GIBCO/BRL) using either 0.5, 2, or 4 μg of plasmid phACTB::FLP (or negative control vector pRevhACTB::FLP) and 0.5 μg of either pFRTZ or pFRTZ-product, as indicated (Fig. 2). β-Galactosidase (β-Gal) activity was detected *in situ* using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (30). Primary EF cultures were plated (5×10^4 cells/ml) in 3.5-cm dishes and transfected by calcium phosphate precipitation (31) with 3 μg of the target pFRTZ or target pNEOβ-GAL (13) followed by X-Gal stain 48 hr later. P19 EC cells were plated (5×10^4 cells/ml) in 10-cm dishes. The next day, pairs of duplicate dishes were transfected by calcium phosphate precipitation (31) with 5 μg of target pFRTZ.2 alone or with 5 μg of phACTB::FLP or pWnt1::FLP as indicated (see Fig. 5). Twenty-four hours later one-half of the dishes were treated with either 0.5 μM of all-*trans* retinoic acid (RA; Sigma) or control diluent for an additional 5 days after which cells were stained with X-Gal.

FIG. 2. Cotransfection assay for Flp function in ES cells. Flp-mediated recombination was detected by the gain of β-Gal activity as assayed by histochemical X-Gal staining (13, 30). (*A*) Positive control transfection (pFRTZ-product). ES cells were transiently transfected with 0.5 μg of pFRTZ-product plus 0.5 μg of phACTB::FLP. Although not necessary for β-Gal activity, phACTB::FLP was included to maintain equivalent amounts of h*ACTB* sequences and DNA between control and experimental transfections. (*B*) Negative control transfection (pFRTZ) included 0.5 μg of pFRTZ and 0.5 μg of the negative control Flp plasmid, pRevhACTB::FLP. (*C*) Experimental transfection (pFRTZ plus phACTB::FLP) contained 0.5 μg of pFRTZ and 0.5 μg of phACTB::FLP. To define a dose-effect relationship, ES cells were transiently transfected with 0.5 μg of pFRTZ (or pFRTZ product) and 0.5, 2, or 4 μg of phACTB::FLP. Following X-Gal staining for β-Gal expression blue-staining cells were counted. Cells staining blue after transfection with pFRTZ-product reflect transfection efficiency. The number of X-Gal-positive cells observed following transfection with pFRTZ plus phACTB::FLP were normalized to the pFRTZ product positive control values. On the basis of this estimation, 30 to 78% of the cells transfected with pFRTZ plus phACTB::FLP underwent a recombination event. Neither pFRTZ, phACTB::FLP or pRevhACTB::FLP generate β-Gal activity when transfected alone.

Transcript Detection. Whole mount *in situ* hybridization to 9.5 days post coitum embryos was performed as described (32) using single-strand digoxigenin-UTP-labeled RNA probes. The *FLP* probe (antisense probe 2, Fig. 1*B*) was a 1386-bp *Eco*RV/*Apa*I fragment from the 3′ end of the *FLP* transgene; control probe (sense) was a 648-bp *Xba*I/*Eco*RV fragment. For Northern blot analyses, fresh tissue or EF cells were homogenized in 6 M guanidinium isothiocyanate and RNA isolated using acid:phenol (33). Total cellular RNA (20 μg) was separated and assayed for hybridization to *FLP* sequence as described (34). Ethidium bromide staining of the gel and filter was used to confirm equivalent RNA loading.

Molecular Analysis of Transgenic Mouse Genotypes. Mouse tails were lysed with NaDodSO$_4$/proteinase K and treated with phenol/chloroform, 1:1 (vol/vol), precipitated with ethanol, and dissolved in 10 mM Tris·HCL, pH 8/1 mM EDTA. For PCR analysis, DNAs were amplified with the following primers: SD42 (5′-GGTCCAACTGCAGCCCAAGCTTCC-3′) and SD41 (5′-GTGGATCGATCCTACCCCTTGCG-3′), for the *FLP* transgene (a 0.75-kb amplified fragment); SD49 (5′-GACTGCTCCAAAGAAGAAGCGTAAGG-3′) and SD68 (5′-GCTATTACGCCAGCTGGCGAAAGG-3′), for the *FRTZ* transgene (a 1.4-kb amplified fragment) and FRTZ-product (a 0.25-kb fragment). The 0.25-kb PCR amplification product was cloned into plasmid pCR (TA cloning, Invitrogen) and sequenced. Genomic DNA isolated from freshly harvested tissues (35) was subjected to *Bam*HI/*Sca*I digestion, and Southern blot analyses. Radiolabeled DNA fragments (specific activity of 2–5 × 10^8 cpm/μg) for use as probes were prepared by random priming (36). Transgene copy number was estimated by including standard amounts of the injected transgene in parallel. Quantitation of radioactivity in specific bands was performed with a Molecular Dynamics PhosphorImager.

RESULTS

Strategy Used to Assay Flp Function in Cell Culture and the Mouse. To generate a test recombination substrate for Flp

function, a *lacZ* gene was disrupted by inserting an FRT cassette that contains stop codons in all three reading frames (24). This target transgene is referred to as *FRTZ*, for FRT-disrupted *lacZ* (Fig. 1*A*). Because the two FRT sequences flanking the cassette are in the same orientation, Flp activity should excise the intervening DNA leaving a single residual FRT in-frame with *lacZ* (Fig. 1*C*). Because there are no ATG codons to initiate translation of functional β-Gal downstream of the FRT cassette, β-Gal activity is strictly dependent on Flp-mediated excisional recombination in a manner similar to previously described β-Gal gain-of-function systems (9, 13).

To broadly express both *FLP* and *FRTZ*, both transgenes were placed under the control of regulatory sequences from h*ACTB*) gene (Fig. 1 *A* and *B*). These h*ACTB* sequences have been shown to be active in most tissues in transgenic mice (22). A "recombined" control transgene, *FRTZ-product*, representing the predicted product of Flp recombination was also constructed (Fig. 1*A*).

Flp-Mediates Efficient Recombination of Extrachromosomal DNA in ES Cells. The efficacy of Flp-mediated excisional recombination in ES cells was tested by assaying for gain of β-Gal activity following cotransfection with target and recombinase plasmids. Cells were transiently transfected with either pFRTZ plus phACTB::FLP, or pFRTZ plus the negative control plasmid pRevhACTB::FLP, followed by X-Gal stain 48 hr later. Positive control cultures were transfected with the "recombined" plasmid, pFRTZ-product (Fig. 2*A*). Cultures transfected with target plasmid pFRTZ, alone or with pRevhACTB::FLP, showed no detectable β-Gal activity (Fig. 2*B*); in contrast, robust activity was observed following cotransfection with phACTB::FLP (Fig. 2*C*).

To estimate recombinase activity, X-Gal-positive cells in each transfection were counted and compared. The number of cells staining blue after transfection with the control "recombined" pFRTZ-product reflected transfection efficiency and, because constitutively active, the maximal number of β-Gal-positive cells. Cotransfection with a fixed amount of target plasmid and increasing amounts of *FLP* expression vector resulted in an increasing percentage of X-Gal-positive cells relative to control pFRTZ-product transfections. A comparison between experimental (pFRTZ plus phACTB::FLP) and control (pFRTZ-product) transfections showed that Flp-mediated β-Gal activation occurred in at least 30% of transfected ES cells and could be as high as 78%. This increase in recombination with increasing Flp-encoding plasmid likely reflects more Flp protein produced per cell, as well as an increase in the proportion of cells that took up both the target and Flp-encoding plasmids (and thereby had the potential to activate *lacZ*).

Flp Can Be Generally Expressed in the Mouse Without Deleterious Effects. To determine whether Flp can function in the mouse and whether Flp expression, itself, would have any adverse effects, mice carrying the h*ACTB*::*FLP* transgene were generated. To identify mouse lines producing Flp in a wide range of tissues, F_1 mice from each founder were screened for ubiquitous *FLP* mRNA and recombinase activity. The distribution and amount of *FLP* mRNA was assessed in the embryo by whole mount *in situ* hybridization and in adult tissues by Northern blot analysis. Two of the five h*ACTB*::*FLP* mouse lines exhibited broad patterns of *FLP* transcripts in 9.5 days post coitum hemizygous embryos (mouse lines 4917 and 4924; Fig. 3 *B* and *D*) and in adult tissues (Fig. 3 *E* and *F*). Flp activity was assayed in EF cultures derived from each transgenic mouse line. The EF cultures were transiently transfected with target plasmid and stained with X-Gal. Maximal Flp activity (approximately 45% of the "recombined" control) was observed in lines 4917 and 4924 (Fig. 3*G*), the same mouse lines that showed broad *FLP* expression (Fig. 3 *B* and *D*). As shown in Fig. 3 *G* and *H*, the amount of recombinase activity detected in EF cultures also correlated with the amount of *FLP* mRNA isolated from each culture. From these experiments it can be inferred that mouse lines 4917 and 4924 are the best candidates for broadly expressed active recombinase. Because no abnormalities were detected in founders or offspring it is likely that Flp activity is nontoxic and can be used in most cell types.

Flp Is Necessary and Sufficient to Recombine Target Sequences in Transgenic Mice. To test whether Flp activity can recombine a chromosomal target *in vivo*, mice carrying *FRTZ* were generated. Five transgenic founders were obtained. F_1 mice from four of the five founders bred as expected for unique single-site integration events (one founder failed to transmit the transgene). Southern blot analysis of liver DNA isolated from each mouse line showed that three of the four mouse lines carried the target *FRTZ* in head-to-tail array: line 4999 carried an array of approximately 4 copies of the *FRTZ* transgene; line 4998, 11 copies; line 5000, 30 copies. Transgene transmission was Mendelian and no rearrangements were observed.

The ability of Flp to catalyze *in vivo* recombination of the target *FRTZ* transgene was initially examined by crossing these mouse lines with the Flp producing lines described above (4917 and 4924). Tail DNA from doubly transgenic animals was analyzed by PCR using primers (diagrammed in Fig. 1 *A* and *B*) specific for detecting either the *FRTZ* transgene, the recombined target *FRTZ-product*, or the *FLP* transgene. Analyses of progeny from three distinct crosses are shown in Fig. 4*A*. The product of Flp-mediated excisional recombination at the *FRTZ* locus, was amplified only in DNA isolated from doubly transgenic mice and was not detected in littermates transgenic for only the recombinase or the target gene. All three *FRTZ* target lines were found to be competent for recombination by this assay. Sequence analysis of the 0.25-kb amplification product showed precise site-specific recombination.

Flp Mediates Recombination in a Variety of Tissues in a Dose-Dependent Manner. The efficiency of Flp recombination at target *FRTZ* loci was assayed by Southern blot analysis. Genomic DNA isolated from doubly transgenic adult mice (target line *FRTZ-4999*; *FLP-4917*) was hybridized with a *lacZ* probe (probe 1, Fig. 1*A*) to allow simultaneous detection of the target *FRTZ* transgene and the product of recombination. As shown in Fig. 4*B*, the new 4.4-kb DNA fragment resulting from the recombined target was present only in samples from doubly transgenic animals, and absent in DNA isolated from either target *FRTZ* (Fig. 4*B*) or *FLP* littermates (data not shown).

The amount of recombination product detected by Southern blot analysis was found to correlate directly with the amount of *FLP* mRNA detected in each tissue by Northern blot hybridization (Fig. 3*E*: lane 6, liver; lane 12, muscle; lane 1, testes). Estimates of recombination efficiency were obtained from phosphorimage quantification of recombined (4.4 kb) to nonrecombined (5.6 kb) bands. In muscle, approximately 30% of the transgenes were in the recombined (4.4 kb) configuration. This represents an average of the actual recombination achieved in the various cell types isolated when dissecting muscle tissue (myofibrils, connective tissue fibroblasts, vascular endothelial cells, lymph node cells, blood cells). The value of 30% therefore represents a low estimate of the maximal recombination efficiency. This frequency is consistent with that observed in the EF cell culture assay derived from the same FLP-4917 mouse line (45%, Fig. 3*G*); indeed, both cell populations showed similar amounts of *FLP* mRNA. Hybridizing with a probe specific to DNA between the FRT sites detected only the unrecombined fragment (data not shown).

A Recombined Transgene Is Stably Transmitted Through the Germ Line. A prerequisite to using Flp to genetically manipulate cell lineages is that the recombination product be stable and heritable. Germ-line transmission of the recombined transgene was demonstrated by outcrossing a doubly transgenic (*FRTZ-5000*; *FLP-4917*) male and genotyping progeny by PCR (data not shown). Both recombined and unre-

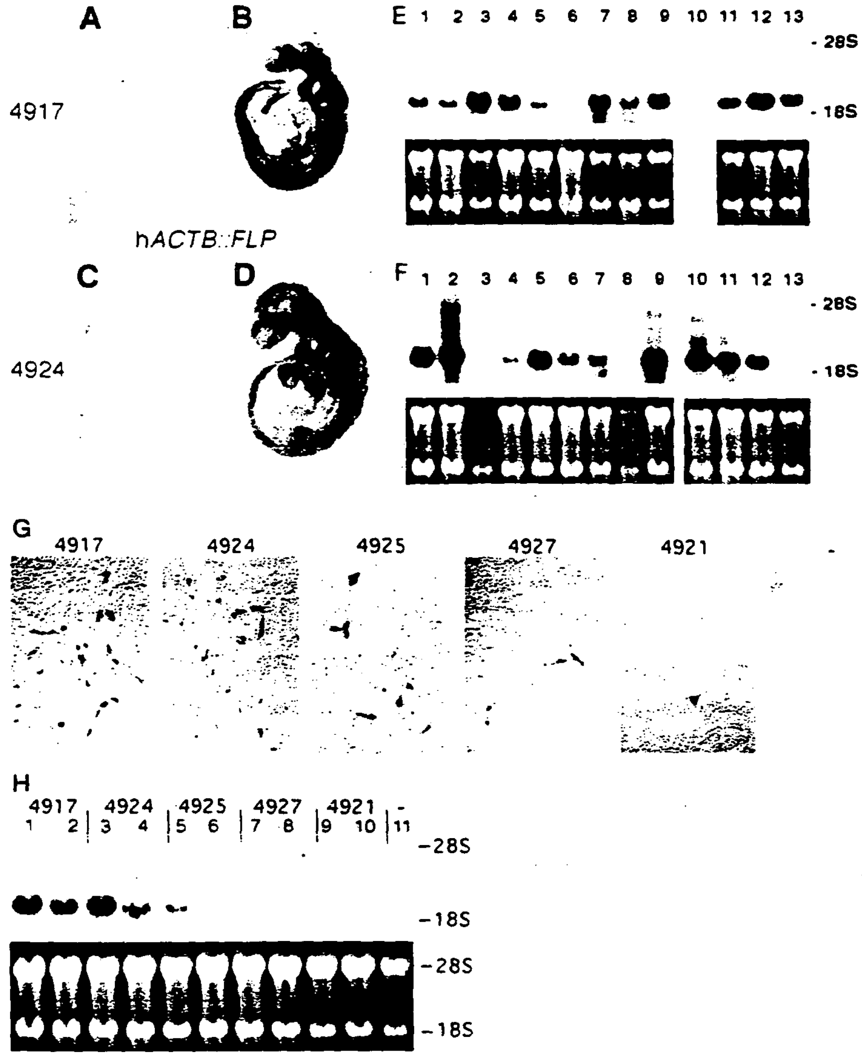

FIG. 3. Human β-actin sequences direct broad expression of *FLP* in the embryo and adult mouse without adverse affects. (*A–D*) Whole mount *in situ* hybridization analysis of *FLP* RNA expression at 9.5 days post coitum. Lateral views of nontransgenic (*A*) and transgenic (*B–D*) embryos from h*ACTB*::*FLP* mouse lines 4917 (*B*) and 4924 (*C* and *D*). (*A*, *B*, and *D*) *FLP* RNA detected using antisense probe 2; (*C*) control sense probe. (*E* and *F*) RNA blot analyses of *FLP* expression in adult tissues from transgenic mouse lines 4917 (*E*) and 4924 (*F*). Total RNA (20 μg) was fractionated by electrophoresis, transferred to nitrocellulose, and assayed for hybridization to ^{32}P-labeled *FLP* probe 3; lower panels show ethidium bromide staining to document RNA loading. (*E* and *F*) Lanes: 1, testes; 2, brain; 3, heart (degraded sample in *F*, therefore repeated in lane 10); 4, intestine; 5, kidney; 6, liver; 7, lung; 8, spleen; 9, ovary; 10, heart (see lane 3 in *E*); 11, quadriceps and hamstring muscles; 12, gastrocnemius and soleus muscles; 13, uterus. Positions of 28S and 18S rRNAs are indicated. (*G*) Assay for Flp function. Primary EF cultures were prepared from hemizygous h*ACTB*::*FLP* transgenic embryos as described (29). Cultures derived from five different transgenic mouse lines (4917, 4924, 4925, 4927, 4921), and one nontransgenic line (–), were transfected with 3 μg of target pNEOβ-GAL (13) followed 48 hr later by histochemical X-Gal stain (30). Maximal activity, as indicated by the number of blue cells, was observed in cultures derived from mouse lines 4917 and 4924. Similar results were obtained following transfection with pFRTZ. (*H*) Expression of *FLP* in EF cultures correlates with activity observed in transfection assay. Total RNA (20 μg) was separated and hybridized to ^{32}P-labeled *FLP* probe 3; ethidium bromide staining of gel in lower panel shows equivalent RNA loading. Two independent cultures from each *FLP* mouse line were analyzed: (lanes 1 and 2) h*ACTB*::*FLP* mouse line 4917, (lanes 3 and 4) line 4924, (lanes 5 and 6) line 4925, (lanes 7 and 8) line 4927, (lanes 9 and 10) line 4921, and (lane 11) nontransgenic negative control.

combined transgenes were detected in this singly transgenic F_3 mouse indicating that recombination was incomplete; a subset of the 30 *FRTZ* transgenes in tandem array underwent recombination.

Conditional Expression of Flp Can Induce Regulated Rearrangement of Target Sequences in Differentiating EC Cells. Controlling expression of the *FLP* transgene is a way to restrict recombination, and therefore gene activation or deletion, to specific cell populations. I investigated whether Flp recombination could be induced in a differentiating EC cell culture system by using *Wnt-1* regulatory sequences (37) to express *FLP* (see Fig. 1*B* for the Wnt1::FLP transgene). RA can induce pluripotent P19 EC cells to differentiate into a mixed population of fibroblasts, astrocytes, and neural cells (38, 39). *Wnt-1* expression is likely induced specifically in neural derivatives, paralleling that seen in embryos where *Wnt-1* mRNA is detected in differentiating neuroectoderm (40).

P19 cells were transiently transfected with target plasmid, target plus phACTB::FLP, or target plus pWnt1::FLP; 0.5 μM RA or control diluent was added to the monolayer 24 hr later.

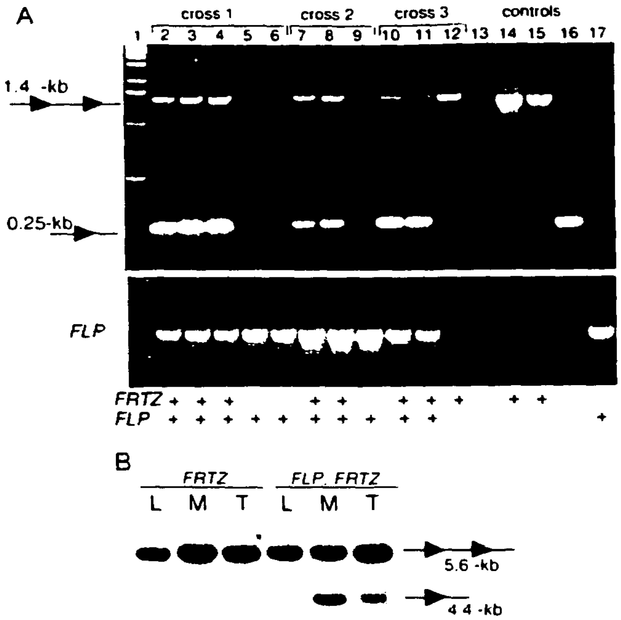

FIG. 4. Dose-dependent Flp recombination in genomic DNA isolated from tissues of doubly transgenic mice. (*A*) Identification of recombined *FRTZ-product* transgenes by PCR amplification of tail DNA. Primers (SD49/SD68) used in this assay flank the FRT-cassette (see Fig. 1*A*): amplification of the target *FRTZ* transgene yields a 1.4-kb fragment and *FRTZ-product*, a 0.25-kb fragment. Parallel reactions using *FLP*-specific primers (SD41/SD42) are shown below. Brackets group littermates from three distinct recombinase target crosses: control single transgenic parental samples and *FRTZ-product* DNA are shown on the right: lanes: 1, 1-kb ladder; 2–6, *FRTZ-5000* × *FLP-4917*; 7–9, *FRTZ-4999* × *FLP-4924*; 10–12, *FRTZ-4999* × *FLP-4917*; 13, no DNA; 14, single transgenic *FRTZ-5000* parental sample; 15, single transgenic *FRTZ-4999* parental sample; 16, *FRTZ-product* DNA; and 17, single transgenic *FLP-4917* parental sample. Genotypes as determined by independent PCR reactions (*FRTZ*, SD23/SD26; *FLP*, SD24/SD25; see Fig. 1) are indicated by plus signs. Sequence analysis of the 0.25-kb product showed precise site-specific recombination (data not shown). (*B*) Correlation between the amount of recombination product and the level of *FLP* RNA expressed in a given tissue. Southern blot analysis using probe 1 (see Fig. 1*A*) of *Bam*HI/*Sca*I-digested genomic tissue DNA (10 μg) isolated from a doubly transgenic (*FLP-4917*; *FRTZ-4999*) mouse or singly transgenic (*FRTZ-4999*) littermate. The expected unrecombined (5.6-kb) and recombined (4.4-kb) fragments within the context of the four-copy array are depicted on the right. Tissue samples include liver (L), muscle (M), and testes (T). For the amount of *FLP* RNA detected in each tissue see Fig. 3*E*: lane 6, liver; lane 12, muscle; lane 1, testes.

Following 5 days of RA treatment, β-Gal activity was assessed by histochemical X-Gal staining. Neural induction was monitored by morphology (the presence of long cellular processes) and culture senescence, as well as by induction of endogenous *Wnt-1* mRNA.

β-Gal activity was detected in target plus pWnt1::FLP cotransfections only following RA induced differentiation (Fig. 5 *C* and *F*). Similarly, endogenous *Wnt-1* expression was absolutely dependent on RA. Low levels of *Wnt-1* transcripts were first detected by Northern blot hybridization after 4 days of RA treatment; no *Wnt-1* RNA was detected in untreated cells (data not shown). As predicted by the nature of the h*ACTB* regulatory sequences, β-Gal-positive cells were observed in the target plus phACTB::FLP cotransfections independent of RA (Fig. 5 *B* and *E*). The target plasmid alone showed no activity (Fig. 5 *A* and *D*). In addition to demonstrating regulated rearrangement of target sequences, these results define a temporal relationship between *FLP* expression and completed recombination. β-Gal activity, reflecting Flp recombination, was observed in the target plus pWnt1::FLP cotransfection within 24 hr of first detecting *Wnt-1* transcripts, and by inference *Wnt1*::*FLP* mRNA.

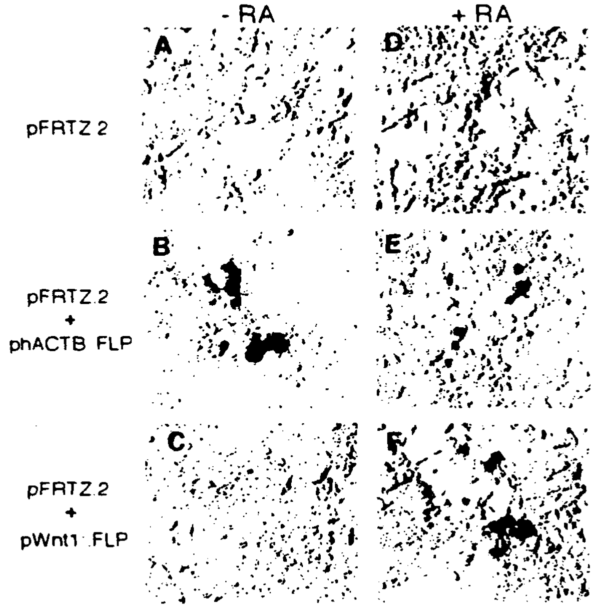

FIG. 5. Regulated Flp recombination in differentiating embryonal carcinoma cells. P19 EC cell monolayers were transiently transfected with the indicated plasmids and induced to differentiate by treatment with RA (38, 39). Target plasmid pFRTZ.2 is identical to pFRTZ except it contains the HSV-tk gene inserted between FRTs. Histochemical X-Gal staining was performed 5 days later. (*A*–*C*) Exposure to control diluent; (*D*–*F*) exposure to 0.5 μM RA.

DISCUSSION

This study demonstrates that Flp can effectively recombine target DNA in ES cells, EC cells, and transgenic mice. I have shown that Flp can direct site-specific and heritable DNA recombination in the mouse, and regulated (inducible) recombination in differentiating EC cells. These properties indicate that Flp can be used to make directed modifications of the mouse genome.

Using this Flp system, recombination of an extrachromosomal target can occur in ES cells with an efficiency similar to that previously observed in mouse embryonal carcinoma (F9) cells (13) and in monkey (CV-1) and human (293) embryonic kidney cells (13, 16). Because the efficacy of Flp recombination estimated here (30–78%) is comparable to that reported for Cre (40–80%; ref. 41), it is likely that this Flp system can be exploited to similarly manipulate ES cell chromosomal DNA. Toward this end, Fiering *et al.* (42) has recently employed a more elaborate two-step selection scheme where Flp-mediated deletion of an integrated selectable marker gene (*PGK-neo*) was reported to occur in 90% of Flp-expressing ES cells.

In the mouse, I have shown that Flp expression is necessary and sufficient for excisional recombination of FRT target sequences. Because recombination was detected at all three chromosomal sites assayed, it is likely that most chromosomal transgenes will be accessible to Flp function. The extent of recombination observed in a given tissue correlated directly with the overall amount of *FLP* mRNA detected in that tissue; it is important to note that this type of tissue analysis presents an average and therefore may underestimate the maximal recombination achieved in a specific cell type. Nonetheless, these results define a dose-effect relationship that suggests that

different degrees of recombination can be attained by varying the strength and specificity of the sequences used to express *FLP*. For some experiments, complete (quantitative) recombination may be needed. The results presented here suggest that one means to achieve this is to increase the level of *FLP* expression. Alternative strategies include identifying Flp variants with higher activity in mammalian cells, or to enhance the nuclear localization of Flp.

The finding that Flp can be generally expressed in the mouse without adverse effects suggests that Flp recombination between random sequences in the mouse genome is rare. If high levels of illegitimate (non-FRT) recombination were occurring due to Flp expression, abnormalities would be expected in *FLP* founders or offspring. No adverse effects were detected. This result suggests that Flp can be used to mediate recombination in a variety of cell types.

Flp-mediated excisional recombination is sufficiently dose sensitive that recombination can be regulated in differentiating EC cells in culture. This was evident from examination of RA-treated P19 cells in which the *Wnt-1* promoter was used to express *FLP*. The temporal induction of *Wnt-1* transcripts following RA-induced differentiation indicates that recombination occurred relatively quickly: *FLP* expression, recombination of the target transgene to reconstitute a functional *lacZ* gene, and subsequent β-Gal production occurred within 24 hr. These results demonstrate that regulated rearrangement of a target sequence can be achieved.

The demonstration that Flp can excise DNA in mice and that the recombination product is heritable, suggests that Flp will be useful to study cell lineages. Considering this potential application, the initial test recombination substrate was designed to indicate and "remember" a recombination event by the irreversible gain of β-Gal activity (dependent only on constitutive promoter activity). Mice transgenic for this target should have the capability of marking cell lineages following introduction of Flp by crossing. Toward this end, mice transgenic for Wnt1::FLP have been generated: by crossing to an "optimal" target mouse, cells originating from the dorsal aspect of the developing central nervous system are predicted to be marked. Although all three *FRTZ* target lines analyzed here were competent for recombination, none of the recombined target alleles were sufficiently active to allow cell marking by X-Gal stain (unpublished observations). The lack of β-Gal activity associated with the observed recombination most likely reflects a position effect on transgene transcription exerted by the genomic integration site since only one in four control *FRTZ-product* mouse lines expresses β-Gal (unpublished observations). Such sensitivity to chromosomal context is also supported by the variation in transcript profiles observed when using the same h*ACTB* regulatory sequences to direct *FLP* expression (two of five lines showed general expression in this study). It is likely that by screening more *FRTZ* target loci, a chromosomal integration site will be identified that can support similarly general *lacZ* expression following Flp recombination.

Together, these findings demonstrate that Flp can serve as a tool to alter the mouse genome. By employing both Flp and Cre, it should be possible to engineer multiple independent recombination reactions (gene activation or deletion events) in mice.

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Attachment F

Expression of Growth Hormone Genes in Transgenic Mice

RICHARD D. PALMITER,* ROBERT E. HAMMER,† AND RALPH L. BRINSTER†
*Howard Hughes Medical Institute
University of Washington
Seattle, Washington 98195
†School of Veterinary Medicine
University of Pennsylvania
Philadelphia, Pennsylvania 19104

OVERVIEW

Human or rat growth hormone (GH) genes have been introduced into all cells of a mouse by microinjection of fertilized eggs but they were not expressed under their own promoters. However, substitution of a mouse metallothionein (MT) promoter allowed expression and regulation comparable to that of the endogenous MT genes. These fusion genes have been used to stimulate the growth of both normal mice and dwarf mice that lack sufficient GH. Substitution of a rat elastase-I promoter directed expression of GH exclusively to the acinar cells of the pancreas. Progress has been made towards developing the hGH gene into a vector that is not expressed in vivo unless an enhancer element is inserted. Recombination between overlapping DNA fragments derived from a MThGH gene, each of which is nonfunctional, has been observed when they are coinjected into mouse eggs. In some cases, functional hGH was produced as evidenced by enhanced growth of the mice.

INTRODUCTION

Growth hormone (GH) is an intermediary in a cascade of hormones that control growth of mammals. It is a single chain polypeptide of 191 amino acids that is synthesized by sommatotroph cells in the pituitary. Hypothalamic hormones, somatostatin, and growth hormone releasing factor, regulate GH synthesis; GH, in turn, regulates the production of insulin-like growth factor-I (IGF-I) by peripheral tissues (Palmiter et al. 1983). GH genes have been isolated from several species (Barta et al. 1981; Seeburg 1982; Gordon et al. 1983). They are composed of five exons and span a total of 2-3 kb; thus, they are of a convenient size for genetic manipulation. They are related to placental lactogen and prolactin genes (Niall et al. 1971).

Several groups have introduced genes into the germline of mice by microinjecting appropriate DNA fragments isolated from plasmids into the pronuclei of fertilized eggs (Gordon et al. 1980; Brinster et al. 1981; Costantini and Lacy 1981; E. Wagner et al. 1981; T. Wagner et al. 1981). With current techniques, about 25% of the mice that develop from this procedure retain the foreign DNA in all of their

cells and transmit them to half of their offspring. Here, we summarize the results obtained with GH genes and indicate some of the future directions.

RESULTS AND DISCUSSION

Metallothionein-growth Hormone Fusion Genes Stimulate Growth of Mice

Because of our initial success in obtaining regulated expression of thymidine kinase gene by fusing the mouse metallothionein-I (MT) promoter to the structural gene isolated from herpes simplex virus (Brinster et al. 1981; Palmiter et al. 1982a), we initiated our experiments with GH in a similar manner. In the first experiments, the MT promoter was fused to the structural gene of rat GH (rGH), and DNA fragments retaining 185 bp of MT promoter and all of the rGH structural gene were microinjected into the pronuclei of fertilized eggs. Of the 21 mice that developed from these eggs, six grew significantly larger than control littermates, and several of these mice had extraordinarily high levels of GH mRNA in the liver and rGH in the serum (Palmiter et al. 1982b). Several lines of mice were started from these transgenic founders. One of these lines, MGH-10, is now in the sixth generation; about 50% of the offspring inherit the chromosome carrying the MTrGH genes and all of these mice grow to about twice the size of normal littermates (Table 1).

These mice grow because the mouse MT promoter causes GH to be synthesized in several organs, notably liver and kidney, instead of in the sommatotroph cells of the pituitary. Although the cellular rate of GH production in these transgenic mice may be lower than in the sommatotroph cells, the enormous size of these organs compared to the pituitary allows serum concentrations to reach levels that are 1000-fold higher than normal (Palmiter et al. 1982b). The production of rGH can be modulated about tenfold by adding zinc, a natural inducer of MT genes, to the diet (Hammer et al. 1984a). However, this extra stimulation of GH synthesis is not required to stimulate growth, presumably because the basal rate of synthesis is sufficient to saturate GH receptors.

Table 1
Effects of MTrGH Gene Expression on Growth and Fertility of Normal (C57 X SJL Hybrids) and Little (lit/lit) Mice[a]

Mice	Males		Females	
	Adult size	Fertility	Adult size	Fertility
Little	15 g	+/-	13 g	++
Little · MTrGH	43 g	++	41 g	+/-
Normal	27 g	+++	23 g	+++
Normal · MTrGH	47 g	++	39 g	+/-

[a]See Palmiter et al. (1982a) and Hammer et al. (1984a,b) for details.

Partial Correction of a Genetic Disease

One of the original attractions of GH genes for expression in transgenic mice was the existence of several mutant strains of mice with defects in GH production. One dwarf strain, called little, grows to about half normal size when homozygous (lit/lit). Although the primary defect that leads to suboptimal GH production in little mice is unknown, injection of GH will stimulate growth (Beamer and Eicher 1976). Therefore, we reasoned that the phenotypic defects in growth could probably be overcome by introducing MTrGH fusion genes into fertilized (lit/lit) eggs. Table 1 shows that enhanced growth was achieved by using this approach (Hammer et al. 1984b).

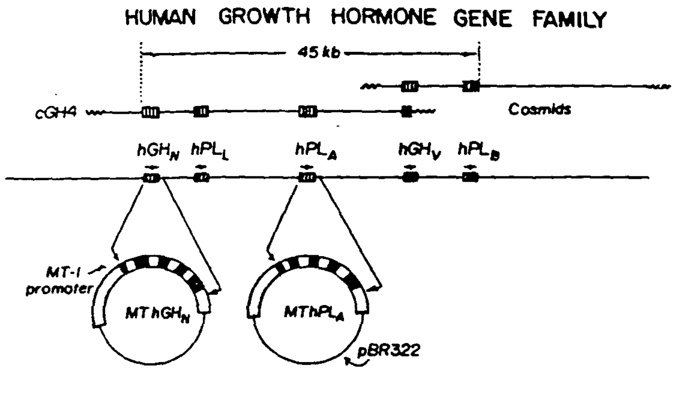
Homozygous (lit/lit) males show a high degree of infertility, whereas females eventually reach full fertility. The fertility of male little mice expressing MTrGH genes was also corrected; all transgenic males have sired at least two litters. However, the fertility of females (either lit/lit or wild-type) that express MTrGH genes was impaired (Table 1 and Hammer et al. 1984b).

Expression of Human Growth Hormone and Placental Lactogen Genes

In humans, there is a cluster of five GH-related genes located within 45 kb of DNA on chromosome 17. These genes have been isolated on two cosmids (Barsh et al. 1983); one of these, cGH4, contains the normal hGH gene, a placental lactogen-like gene (hPL_L) and a normal placental lactogen gene (hPL_A). None of sixteen transgenic mice carrying this cosmid showed enhanced growth, serum hPL, or hGH as measured by RIA (Fig. 1). Another 16 transgenic mice with plasmids containing either hGH or rGH genes also failed to express these genes (Hammer et al. 1984b). Thus, it appears that the signals necessary for proper expression of human or rat GH genes are either absent from the DNA molecules tested thus far or they are incapable of responding to mouse regulatory factors when introduced into the germline by microinjection.

We have achieved expression of hGH_N and hPL_A by fusing these structural genes to the mouse MT promoter in a manner similar to that used for rGH (Fig. 1). MThGH fusion genes work as well as MTrGH genes at stimulating the growth of mice (Palmiter et al. 1983). The foreign gene is expressed predominantly in liver, heart, testis, and intestine; but measurable levels of MThGH mRNA are detectable in other tissues as well. The pattern of expression resembles that of the endogenous MT genes (Palmiter et al. 1983).

One line of mice expressing a high level (about 7 μg/ml in the serum) of hPL_A has been examined in detail to see if this hormone has any effect upon murine growth or reproductive physiology. The fertility of males and females expressing this gene is normal; fetal and adolescent growth are normal; and maternal behavior and lactation are normal. Thus, we have been unable to help unravel the mystery concerning the physiological role of hPL (Chard 1983).



DNA	EXPRESSION mRNA	Protein	Growth
cGH4	+/-	−	−
MThGH$_N$	+	+	+
MThPL$_A$	+	+	−

Figure 1
Organization of the human GH gene family and expression of hGH$_N$ and hPL$_A$ in transgenic mice. Cosmid, cGH4, and two hybrid genes with the mouse MT-I gene promoter fused to the structural gene of hGH$_N$ or hPL$_A$ were microinjected into mouse eggs. Expression of these genes in transgenic mice was tested by monitoring growth, hGH and hPL mRNA levels, and by radioimmune assay of hGH and hPL in serum samples. Very low levels of hGH mRNA were detected in liver and pituitary samples from a few mice carrying the cGH4 cosmid DNA, but no serum proteins corresponding to hGH or hPL were detectable. See Palmiter et al. (1983) for details regarding MThGH$_N$ gene expression.

Development of the hGH Gene into an Enhancer Vector

To help define the DNA sequences involved in cell-specific gene expression, it would be useful to have a gene that is not expressed at all in vivo unless appropriate sequences, so-called enhancer elements, are supplied. If the vector coded for a gene product that had a pronounced physiological effect when expressed in any cell type, then one could test various enhancers in a common vector. Expression could initially be monitored by the physiological effect and then the source of the gene product could be tracked to the cell type of origin. The data presented above suggested that GH genes might be adaptable to this purpose since they were not expressed under their own promoters but produced a readily apparent physiological effect when expressed under control of a heterologous promoter. An advantage of GH over many other secreted hormones is that the only post-translational modification involves removal of the signal peptide. Thus, it should be possible to produce and secrete functional GH in any cell type with signal peptidase.

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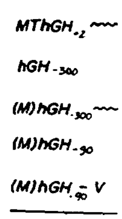

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Our approach was to introduce DNA fragments containing enhancer function upstream of the hGH promoter. We chose the mouse MT-1 gene region located between -46 and -185, which includes the entire cluster of metal regulatory elements (MREs), as a test enhancer (Stuart et al. 1984). The various constructs were tested in tissue culture cells by standard transfection and transient assay or by microinjecting them into fertilized eggs and testing for expression in the liver of fetuses or adults. Figure 2 shows that the tissue culture assay was somewhat permissive and allowed a low level of expression (measured as hGH mRNA) whereas the in vivo test system was more stringent and no expression could be detected in the absence of the test enhancer. Insertion of the MRE enhancer region (Fig. 2, solid box) about 300 bp upstream of the hGH cap site had no effect. Likewise, several other enhancers, including the SV40 72-bp repeats, had no effect in vivo when inserted at -300 (data not shown). However, when the MRE region was moved to within 90 bp of the hGH cap site, then expression in tissue culture cells could be detected and hGH mRNA was inducible by metals. However, this construct was still ineffective in vivo unless the vector sequences were removed. The control (hGH_{-90}) without vector sequences is inactive in vivo. We are in the process of testing other enhancers to see if this construct has general utility as an enhancer vector. It appears that DNA sequences lying between -90 and -300 of the hGH gene prevent MRE enhancer activation of hGH transcription and that this gene is very sensitive to vector (pUC) sequences in vivo.

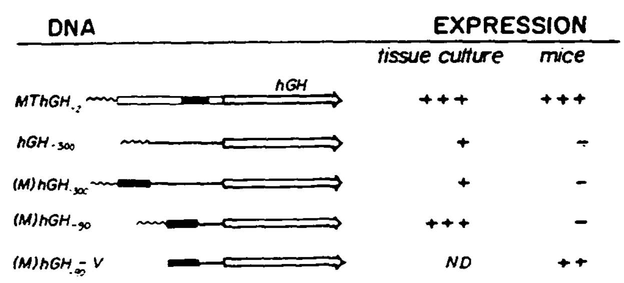

Figure 2
Development of an hGH enhancer vector. MThGH is a hybrid gene with the mouse MT-I promoter fused to the hGH structural gene (arrow) at the BamHI site (+2) as shown in Figure 1; the solid box represents the metal regulatory elements (MRE; -46 to -185) of the mouse MT-I gene; and the wavy line represents pUC vector sequences. hGH_{-300} consists of the hGH gene with about 300 bp of 5' flanking sequence. (M)hGH_{-300} has the MRE inserted at -300. (M)hGH_{-90} has the MRE inserted at -90. The last construction lacks vector (V) sequences. To monitor expression, DNA was either transfected into baby hamster kidney cells and hGH mRNA was measured 48 hr later, or DNA was microinjected into mouse eggs and hGH mRNA was assayed in fetal or adult liver.

Use of hGH to Study Cell-specific Gene Expression

The rat elastase-I gene is expressed in the acinar cells of the pancreas. When this gene was introduced into the germline of mice, rat elastase was still expressed almost exclusively in the pancreas (Swift et al. 1984). Deletion of all but 205 bp of 5′ flanking sequences still allowed pancreas-specific expression but the absolute level of expression was somewhat lower (Fig. 3). To determine whether 5′ rat elastase sequences were sufficient to direct expression to the pancreas, we inserted a convenient linker at +8 of rat elastase gene and fused the 5′ flanking sequences to the +2 position of hGH. This fusion gene was also expressed in a pancreas-specific manner when either 4500 bp or 205 bp of rat elastase sequences were present (Fig. 3). The level of expression of either rat elastase or hGH was at least three orders of magnitude higher in pancreas than in any other tissue tested and in many cases it was five orders of magnitude higher (Swift et al. 1984; Ornitz et al. 1985). Furthermore, the level of foreign mRNA produced frequently exceeded 10^4 molecules/cell. We do not yet know whether the enhancer and promoter functions are separable, but if so then it appears that the elastase promoter is very tightly controlled by its associated enhancer.

It is interesting to note that the transgenic mice expressing the elastase-hGH fusion genes did not show any signs of enhanced growth despite the high level of expression in the pancreas. Immunofluorescent analysis of hGH in sections of the pancreas from these animals revealed intense fluorescence over the acinar cells and in the collecting ducts; but islets, lymph nodes, and capillaries were negative (Ornitz et al. 1985). Thus, we suspect that hGH was secreted along with the digestive enzymes into the gut and none was resorbed intact or secreted into the circulation. In fact, the absence of a growth effect argues strongly that these mice were not synthesizing hGH in any tissue that secretes into the bloodstream. This is one limitation of hGH as the ideal enhancer vector as discussed in the previous section.

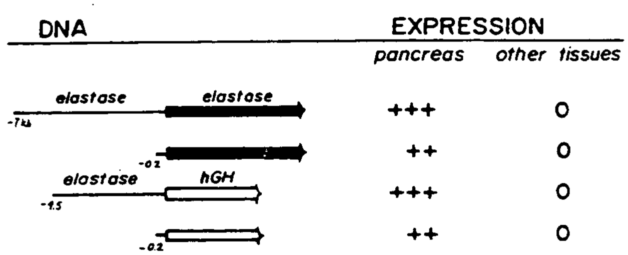

Figure 3
Tissue-specific expression of rat elastase and elastase-hGH fusion genes. The rat elastase-I gene with 7.0 kb or 0.2 kb of 5′ flanking sequence was expressed almost exclusively in the pancreas (Swift et al. 1984). Hybrid genes with 4.5 kb and 0.2 kb of elastase 5′ flanking sequences fused to the hGH structural gene were also expressed exclusively in the pancreas (Ornitz et al. 1985).

Use of MThGH to Study Recombination

One of our goals is to obtain some control over the integration of foreign DNA. At present we have little control over the number of copies that integrate or the site at which they integrate. The integration frequency is improved about fivefold by injecting linear molecules compared to circular forms (Brinster et al. 1985). There is usually a single integration site and if there is more than one copy of the foreign DNA integrated they are usually in a tandem head-to-tail array. The tandem arrays could result from homologous recombination between the injected molecules either before or during the integration process (Brinster et al. 1981). However, we have not observed homologous recombination between the injected DNA and homologous endogenous genes (R. Palmiter et al., unpubl.).

We constructed the MThGH vectors shown in Figure 4 as a means of studying recombination. First, we inserted a FLP sequence from the 2-micron circle of yeast into the third intron of MThGH (Fig. 4, solid circle) because an enzyme (FLPase) that will promote site-specific recombination at this sequence can be isolated (Cox 1983; Meyer-Leon et al. 1984). This enzyme may ultimately be useful for targeting foreign DNA to specific sites. Insertion of the FLP sequence had no effect on MThGH expression (Fig. 4). Then we deleted several hundred base pairs between *Nar*I and *Sma*I (plasmid #165), which should result in a truncated hGH protein of 104 amino acids. We have not yet looked for this protein, but we know that mRNA levels are high and that the mice do not grow larger than normal; we suspect that

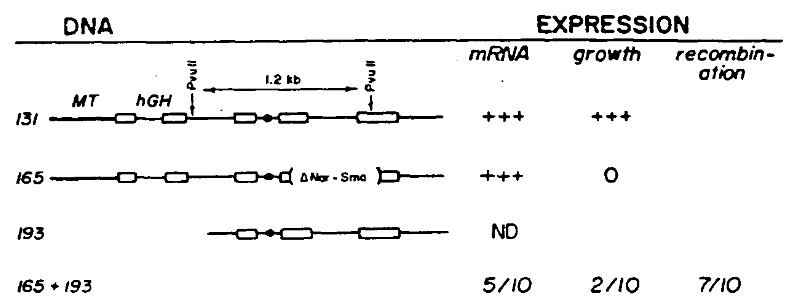

Figure 4
MThGH constructions designed to test for recombination. Plasmid #131 (top line) is a MThGH fusion gene as shown in Figure 1 with the addition of a FLP sequence (•) in the third intron. Plasmid #165 has a deletion between the *Nar*I and *Sma*I sites which deletes the last 87 amino acids from hGH and the 3' *Pvu*II site. Fragment #193 lacks the MT promoter, the first two exons of hGH, and the 5' *Pvu*II site. Coinjection of a few hundred copies of #165 and #193 into fertilized eggs resulted in ten transgenic mice: seven of them showed evidence of the correct 1.2-kb *Pvu*II fragment; five expressed RNA sequences present only in #193; and two of them grew significantly larger than controls.

this protein is made but is biologically inactive. The ultimate experiment will be to supply the correct information to replace the deleted nucleotides by introducing DNA fragment #193 along with FLPase into eggs from mice carrying resident copies of fragment #165. As a control for this experiment, we coinjected fragments #165 and #193. Ten mice resulting from this experiment retained these plasmid sequences. Southern blots of their DNA revealed the presence of a 1.2 kb *Pvu*II fragment that is indicative of recombination between the two DNA molecules. Five of these mice had mRNA sequences corresponding to the region deleted from fragment #165, and two of them grew significantly larger than their control littermates. Recombination between DNA molecules that are introduced into cells simultaneously has been reported (Folger et al. 1982; DeSaint Vincent and Wahl 1983; Shapira et al. 1983; Small and Scangos 1983; Subramani and Berg 1983), but in most of those experiments there was selection for the recombination event. In this experiment there was no selection, yet the frequency of recombination was about 70%. Furthermore, in two of these cases the recombination occurred in a manner that allowed production of functional hGH. In the other three mice that showed evidence of gene expression but failed to grow larger than normal, the recombination events may have been imprecise and thereby resulted in an aberrant hGH protein.

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Attachment G

Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 4223–4227, July 1983
Biochemistry

The FLP protein of the yeast 2-μm plasmid: Expression of a eukaryotic genetic recombination system in *Escherichia coli*

(gene expression/site-specific recombination/gene cloning)

MICHAEL M. COX*

Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

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ABSTRACT The *FLP* gene of the yeast 2-μm plasmid is involved in a site-specific recombination event that results in the inversion of a set of sequences within the plasmid. This gene has been cloned and expressed in *Escherichia coli*. Expression of the *FLP* gene results in efficient recombination within the bacterial cell, which is specific for plasmids containing at least one 2-μm plasmid recombination site. This work demonstrates that (*i*) FLP protein is actively involved in 2-μm plasmid recombination; (*ii*) no other factors specific to yeast are required for the reaction; (*iii*) FLP protein acts efficiently in *trans*; (*iv*) FLP protein will promote site-specific insertion and deletion reactions in addition to the inversion reaction; and (*v*) FLP-promoted recombination is not dependent upon any DNA structural features unique to yeast chromatin.

The yeast 2-μm plasmid is a circular DNA molecule of 6,318 base pairs (bp) present at about 70 copies per cell in most yeast strains (1). Its sequence has been determined (2), and it has been used in a variety of ways as a yeast cloning vector (3). A prominent feature of the sequence is the presence of a 599-bp inverted repeat. The two copies of this sequence are present on opposite sides of the circle. Recombination between these repeats, mediated by a system encoded within the plasmid, serves to invert a segment of the plasmid sequence relative to the remainder. This results in two forms of the plasmid, A and B (Fig. 1), which are found in equal concentrations in yeast cells (1).

This recombination event has been shown to be site specific. The part of the repeat that is required for recombination has been narrowed to a region of 65 bp spanning an *Xba* I restriction site (4). A 4-bp deletion within the *Xba* I site abolishes recombination, so that this site is clearly part of the required sequence (4). This system is thus analogous to the site-specific DNA inversions, deletions, and insertions that occur in a number of prokaryotic systems (5–8). No cellular function has been defined for 2-μm plasmid circle recombination. This system, however, offers an opportunity to examine a eukaryotic recombination event.

The 2-μm plasmid contains a number of open reading frames (1, 2). One of these, designated *FLP*, has been implicated in the recombination process (9, 10). It has not been determined whether other yeast factors are necessary, although the other genes on the plasmid are not required (4). The FLP gene product may carry out the reaction by itself or play an auxiliary role as part of a larger complex. In any case, this gene provides the obvious starting point for an analysis of the system. To provide a potentially rich source of the FLP protein, it seemed desirable to clone and express the *FLP* gene in *Escherichia coli*. This has been accomplished and has permitted an analysis of the properties of 2-μm plasmid recombination in an *in vivo* system in the absence of other yeast proteins.

MATERIALS AND METHODS

Enzymes and Plasmid DNA. *E. coli* DNA polymerase I and T4 DNA ligase were gifts from S. Scherer of this department. Nuclease S1 was purchased from Sigma. Restriction endonucleases were purchased from New England BioLabs. The plasmid 82-6B was a gift of V. Zakian of the Fred Hutchinson Cancer Research Center (Seattle, WA). The plasmid pCQV2 (11), constructed by C. Queen of the Massachusetts Institute of Technology, was provided by J. Flynn of this department. The plasmid pXF3 (12) was provided by P. Southern of this department.

Miscellaneous Methods. Isolation of DNA fragments from agarose gels, restriction digests, DNA ligations, and transformation of bacterial cells with plasmid DNA were carried out by procedures described in detail elsewhere (12, 13). Rapid screening of transformed colonies was carried out by using the rapid plasmid isolation from small cultures described by Davis *et al.* (13). For more detailed analysis, including the results presented in Figs. 3–6, plasmid DNA was purified by banding twice in cesium chloride gradients containing ethidium bromide as described (12). In every case this DNA was obtained from cells grown with selection for all appropriate drug resistances. Agarose gel electrophoresis was carried out by using 0.8% agarose in a Tris acetate buffer system as described (13).

Strains. Recombinant plasmids in all cases were recovered in the *E. coli* strain HB101 ($recA^-$) (12). The plasmid pCQV2 was maintained in *E. coli* C600 ($recA^+$) (12).

Plasmid Constructions. Plasmids constructed in this study are illustrated in Fig. 2.

FLP substrates. The plasmid 82-6B was isolated by J. E. Donelson from a library of random yeast DNA fragments cloned in the *E. coli* plasmid pMB9. It contains about 1.5 copies of the yeast 2-μm plasmid sequence (2). A full-length copy of the A form of the 2-μm plasmid was obtained by isolating the smaller of the two fragments generated by *Pst* I cleavage of plasmid 82-6B. This was inserted into the *Pst* I site of pXF3 to produce the plasmid pMMC1. The plasmid pXF3 contains no *Ava* I site. The plasmid pMMC1 was digested with *Ava* I and religated, and a clone was selected in which the short 1.5-kilobase (kb) fragment had been reinserted in the opposite orientation. This was designated pMMC3 and contains the two 2-μm plasmid repeats in direct orientation. The plasmid pMMC10 was con-

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Abbreviations: bp, base pair(s); phage λp_R, bacteriophage λ rightward promoter; kb, kilobase(s).

* Present address: Dept. of Biochemistry, College of Agricultural and Life Sciences, Univ. of Wisconsin-Madison, 420 Henry Mall, Madison, WI 53706.

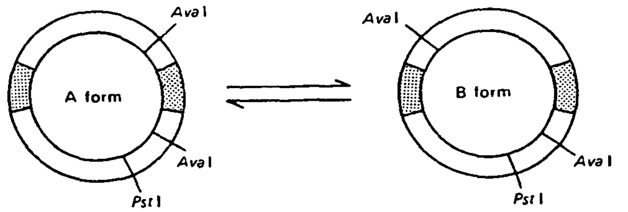

FIG. 1. FLP-promoted recombinational inversion within the yeast 2-μm plasmid. The inverted repeats are identified as the shaded regions.

structed by digestion of 10 μg of pMMC3 DNA with *Xba* I, isolation of the larger of the two fragments produced, and circularization of that fragment with T4 DNA ligase.

FLP expression. The plasmid pCQV2 (11) contains sequences from pBR322, including the replication origin and the region encoding ampicillin resistance. The plasmid also contains sequences from bacteriophage λ, including the temperature-sensitive repressor *c*I857, the phage λ rightward promoter (λp_R), and sequences downstream up to and including the initiation codon of the *cro* gene. The pCQV2 fragment between the *Sph* I and *Pvu* II sites was replaced with the *Sph* I–*Pvu* II fragment from pMMC1, which spans the *FLP* gene and includes one complete 2-μm plasmid repeat (Fig. 2). This generated a new plasmid, pMMC4, in which the *FLP* gene was present in the proper orientation relative to λp_R, but was about 100 bp downstream from the normal *cro* start site. To remove this 100 bp, 3 μg of pMMC4 DNA was cleaved with *Sph* I and *Bam*HI, treated with 0.2 unit of nuclease S1, and recircularized with T4 DNA ligase. Analysis of the products of this procedure is described in *Results*.

RESULTS

Expression of the FLP Protein in *E. coli*. The sequences after the *cro* initiation codon in pCQV2 were reconstructed so that a *Bam*HI site is present. Cleavage with *Bam*HI and removal of the four-nucleotide overhang leaves a blunt end immediately after the *cro* ATG.

The *FLP* gene has a cleavage site for *Sph* I (used in the construction of pMMC4) located near its initiation codon (2). Cleavage with *Sph* I and removal of the four-nucleotide overhang leaves a blunt end, which eliminates only the initiation codon. Thus, blunt-end ligation to the prepared *Bam*HI site in pCQV2 should result in a construction in which the *FLP* gene is positioned exactly as the *cro* gene normally is positioned relative to phage λp_R and the cro ribosome binding site.

Purified pCQV2 DNA (Fig. 3) showed a normal gel pattern consisting primarily of supercoiled monomers. The lower of the two minor bands in Fig. 3 reflects contamination by the relaxed monomers present in small amounts in all such preparations. The other minor band probably represents a small number of supercoiled dimers generated by homologous recombination. Strain C600, from which the pCQV2 DNA was obtained, is *recA*$^+$.

Purified pMMC4 DNA also showed a normal gel pattern (Fig. 3) except for the presence of a minor band migrating at approximately the position expected for supercoiled tetramers. A possible explanation for this band will be presented in the *Discussion*.

To place the *FLP* gene in the position normally occupied by the *cro* gene relative to phage λp_R, pMMC4 DNA was treated as described. The DNA was recovered in HB101, and the plasmid DNA from 30 of the resulting amp^R colonies was subjected to a rapid screening. In each case the recovered plasmids appeared to be slightly shorter than pMMC4, as expected (not shown). In six cases, however, only a small fraction of the DNA appeared as monomers, the remainder migrating as a series of larger bands. The patterns were identical in all six cases. One of these was designated pMMC6 and used for further study.

The plasmid pMMC6 was purified from cells grown at 30°C, and the DNA gel pattern (Fig. 3) was consistent with a series of multimeric species, with dimers and tetramers most prominent. This result might be expected if the *FLP* gene were expressed and if the FLP protein actively promoted recombination involving the recombination site within the 2-μm plasmid repeat inherited by pMMC6 from pMMC4.

This explanation for the observed gel pattern is supported by

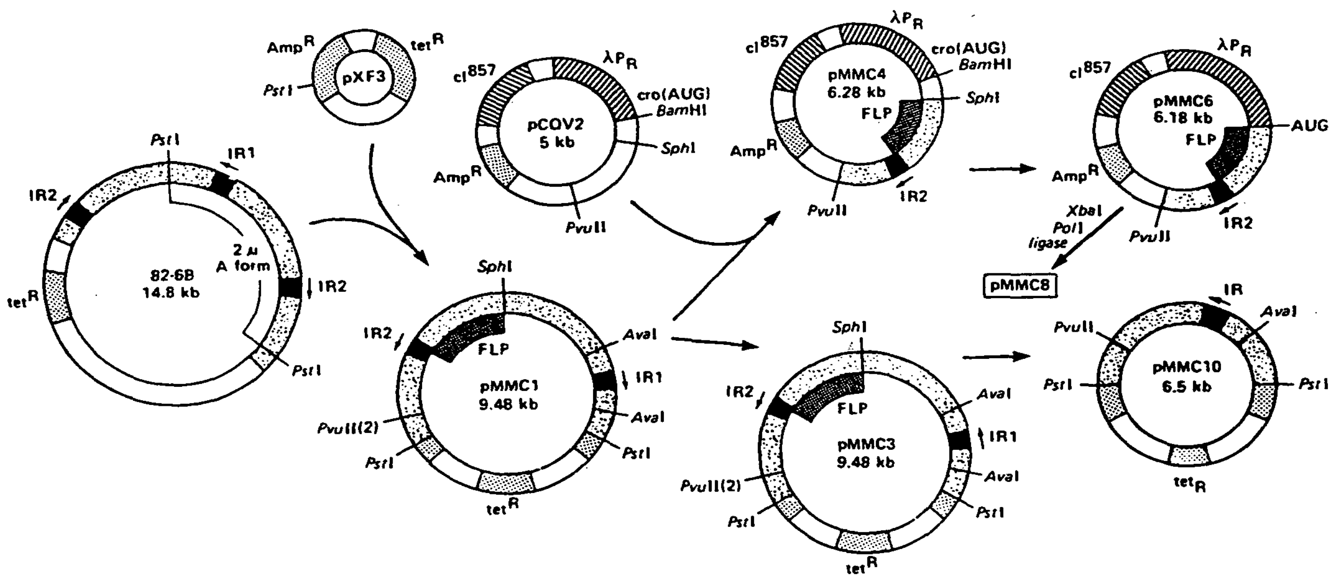

FIG. 2. Construction of recombinant plasmids used in this work. The relative size of the plasmids and the position and size of various sequences are approximate. Sequences derived from different sources (bacteriophage λ, the 2-μm plasmid, etc.) are shaded differently. IR, inverted repeats (▬) from the 2-μm plasmid.

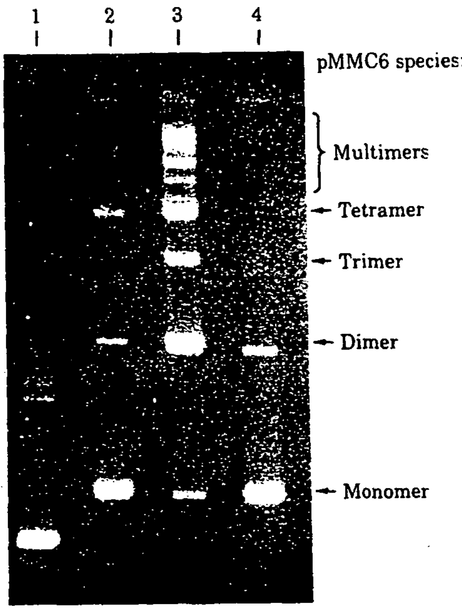

FIG. 3. Expression of FLP protein in *E. coli*. Agarose gel electrophoresis of plasmid DNAs was carried out as described. Lanes: 1, pCQV2; 2, pMMC4; 3, pMMC6; 4, pMMC8.

several additional observations.

(*i*) Cleavage of pMMC6 DNA with *Pvu* II, which should cleave both pMMC4 and pMMC6 only once, resulted in a single band (Fig. 4). Thus, all of the bands are derived from a single DNA species and are not artifacts of the purification procedure.

(*ii*) Monomeric pMMC6 DNA was isolated from agarose gels as described by Dretzen *et al.* (14). The recovered DNA yielded a single band on agarose gels that migrated in the position of supercoiled pMMC6 monomers. In addition, cleavage of the DNA with *Pvu* II yielded the single band expected for cleavage of pMMC6 DNA. Aliquots of a single culture of HB101 were

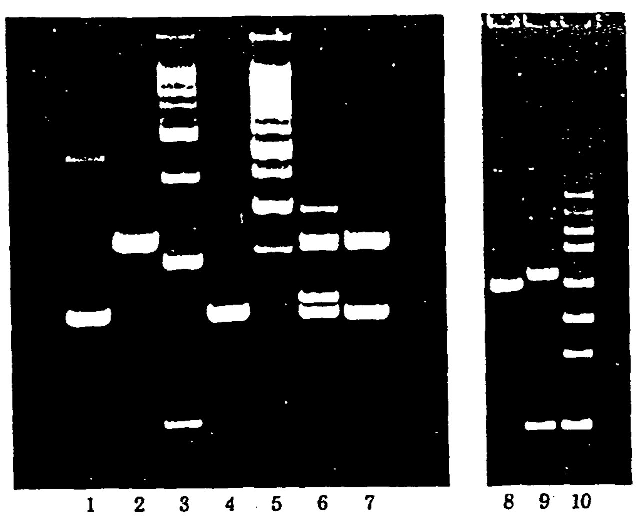

FIG. 4. Intermolecular recombination promoted by FLP protein. Agarose gel electrophoresis and restriction digests were carried out as described. Lanes: 1, pMMC3 DNA; 2, *Pvu* II-digested pMMC3 DNA; 3, pMMC6 DNA; 4, *Pvu* II-digested pMMC6 DNA; 5, pMMC6/3 DNA; 6, *Pvu* II-digested pMMC6/3 DNA; 7, *Pvu* II-digested pMMC3 and pMMC6 DNAs; 8, *Pst* I-digested pMMC6 DNA; 9, *Pst* I-digested pMMC3 DNA; 10, *Pst* I-digested pMMC6/3 DNA.

transformed with pMMC6 monomers or pMMC4 or pCQV2 in parallel experiments. Six colonies from each transformation were subjected to rapid screening. In each case, DNA from cells transformed by pCQV2 or pMMC4 exhibited the primarily monomeric pattern shown in Fig. 3. When the same cells were transformed with pMMC6 monomers, however, the multimeric pattern of Fig. 3 was always reproduced. This pattern is thus not an artifact related to the cells used to recover the plasmid.

(*iii*) Sequences within the 2-μm plasmid repeat that are known to be required for recombination were altered in pMMC6. This is accomplished most easily by destroying the *Xba* I site as demonstrated by the work in yeast (4). In this case purified pMMC6 DNA was cleaved with *Xba* I, the resulting overhang was filled in with DNA polymerase I, and the products were recircularized with T4 DNA ligase. The only *Xba* I site in pMMC6 is the one within the 2-μm plasmid repeat. Several transformants were subjected again to rapid screening. Only supercoiled monomers were evident in all cases in which the *Xba* I site had been successfully destroyed without affecting the size of the plasmid (as determined by gel electrophoresis). One of these was designated pMMC8; purified pMMC8 DNA occupies lane 8 in Fig. 3. Destruction of the *Xba* I site clearly prevented formation of the multimeric pattern. This experiment directly implicates the *FLP* system in the generation of multimers. The end of the open reading frame that defines the *FLP* gene and the *Xba* I site are separated by 183 bp (2). Experiments described below demonstrate that whereas pMMC8 cannot participate in FLP-promoted recombination, it does express an active FLP protein.

FLP-Promoted Intermolecular Recombination. The pMMC6 multimers are most easily explained by intermolecular recombination events. It is possible, however, that the multimers could arise from intramolecular recombination within replication intermediates or "θ" structures. To detect intermolecular reactions, pMMC6 monomers and pMMC3 were used to cotransform HB101 simultaneously to amp^R and tet^R. The DNA isolated from cotransformed cells was designated pMMC6/3. Each of the plasmids alone yielded a single band when cleaved with *Pvu* II (Fig. 4). The gel pattern observed for purified pMMC6/3 DNA differed from that expected for a simple mixture of the two. When cleaved with *Pvu* II, pMMC6/3 yielded not two but at least five bands. Extensive overdigestion with *Pvu* II did not alter this pattern.

To rationalize this pattern, arbitrarily designate the recombination sites in pMMC3 as 1 and 2. The products of recombination between pMMC6 and pMMC3 should yield new *Pvu* II fragments of 9.37, 9.22, 6.29, and 6.43 kb if the pMMC3 sites are equivalent. Two more fragments of 9.48 and 6.18 kb are expected from the unreacted plasmids. At least two bands were evident in the pMMC6/3 *Pvu* II pattern for each of the bands for the cut, unreacted plasmids (Fig. 4). The presence of two bands rather than three in each case could mean that (*i*) intermolecular recombination proceeded to a point where no unreacted plasmids remained, (*ii*) the two recombination sites in pMMC3 are not equivalent, or (*iii*) the extra bands were simply not resolved on this gel. Longer gels and use of less pMMC6/3 DNA failed to reveal the additional bands (not shown).

An additional restriction digest was carried out, therefore, with results again shown in Fig. 4. The plasmids pMMC6 and pMMC3 have one and two *Pst* I restriction sites, respectively, with the digestion patterns shown in Fig. 4. The larger of the two pMMC3 fragments corresponds to the complete 2-μm plasmid sequence present in this plasmid and contains both recombination sites. This fragment was present only as a very minor species in the *Pst* I digest of pMMC6/3, indicating that explanation *i* above is probably correct. The largest band in the

Pvu II digest of pMMC6/3 could result from recombination between two pMMC3 molecules involving site 1 in one molecule and site 2 in the other. This would yield a hybrid dimer with *Pvu* II fragments of 12.4 and 6.4 kb. Thus, all of the observed bands are easily explained by simple, bimolecular reactions. The FLP protein expressed by pMMC6 appears to promote intermolecular recombination at some frequency, and, in fact, this type of reaction is very probable under the conditions prevailing in pMMC6/3-transformed cells at 30°C. These experiments do not preclude the possibility that intramolecular recombination may occur during replication.

Recombinational Inversion in *trans*. A similar experiment was carried out to determine if the FLP protein from pMMC6 could promote a recombinational inversion analogous to the normal FLP-promoted recombination event that occurs in yeast. The experiment also was designed to determine if the protein could act in *trans*. HB101 was cotransformed with pMMC8 and pMMC1. Plasmid DNA purified from cotransformed cells was designated pMMC8/1. Within the pMMC8/1 gel pattern, bands derived from pMMC8 were apparent (Fig. 5). This plasmid, which lacks the *Xba* I sequence required for 2-μm plasmid recombination, was recovered intact from the cotransformed cells. The normal pMMC1 bands were not present, however, but were replaced by a number of bands corresponding to larger DNA species. Cleaving pMMC8/1 with *Xba* I yielded the intact pMMC8 bands and the two fragments expected from pMMC1, demonstrating that these larger species are derived from pMMC1. The larger pMMC1 species appeared only in cells cotransformed with pMMC8; therefore, they must reflect FLP-promoted recombination. These results imply that FLP protein is expressed by pMMC8. Because pMMC8 DNA was not affected, the recombination events were clearly dependent upon an intact 2-μm plasmid recombination site. In addition, the FLP protein in this experiment must act in *trans*.

Because the *Xba* I site lies within the sequences required for recombination, this experiment will not detect an intramolecular inversion (Figs. 1 and 2). To determine if inversions had taken place, the pMMC8/1 DNA was cleaved with *Ava* I. This enzyme did not affect pMMC8 but cleaved pMMC1 (which contains one complete 2-μm plasmid sequence in the A form) into two fragments of 1,506 and 7,972 bp. Conversion of the 2-μm plasmid sequence in pMMC1 to the B form would be indicated by the presence of *Ava* I fragments of 6,098 and 3,380 bp (2) (see Figs. 1 and 2). As shown in Fig. 5, cleavage of pMMC8/1 with *Ava* I yielded the bands expected for intact pMMC8, two bands expected for pMMC1 (A form), and two new bands in the positions expected for pMMC1 (B form). This DNA concentration was chosen so that the larger bands could be clearly distinguished. The 1.5-kb band from pMMC1 (A form) was present but stained weakly compared to the others. Its presence was confirmed by using larger amounts of the cleaved DNA (not shown). The largest bands from both pMMC1 (A form) and pMMC1 (B form) appeared to be present in equal or nearly equal concentrations. This suggests an efficient inversion reaction that had proceeded to equilibrium or near equilibrium. Because the inversions are also dependent upon the presence of pMMC8, all of these events must be mediated by FLP protein from pMMC8 acting in *trans*.

Recombinational Deletion in *trans*. The third and final type of site-specific recombination is a deletion event that may occur when two sites are present on the same DNA molecule in the same orientation. To detect such an event in this system, HB101 was cotransformed with pMMC8 and pMMC3. The plasmid pMMC10, which is equivalent to one of the expected products of an FLP-promoted pMMC3 deletion, was also used in the cotransformation of HB101 with pMMC8 for control purposes. Purified DNA from the two sets of cotransformed cells was designated pMMC8/3 and pMMC8/10, respectively. The gel pattern observed with pMMC8/10 DNA again showed pMMC8 recovered intact (Fig. 6). The plasmid pMMC10, which contains one 2-μm plasmid repeat, was found as the larger DNA species resulting from FLP-promoted recombination as described above. Cleavage with *Xba* I reduced these larger species to the single band expected for pMMC10, confirming that these species are derived from pMMC10. The pattern ob-

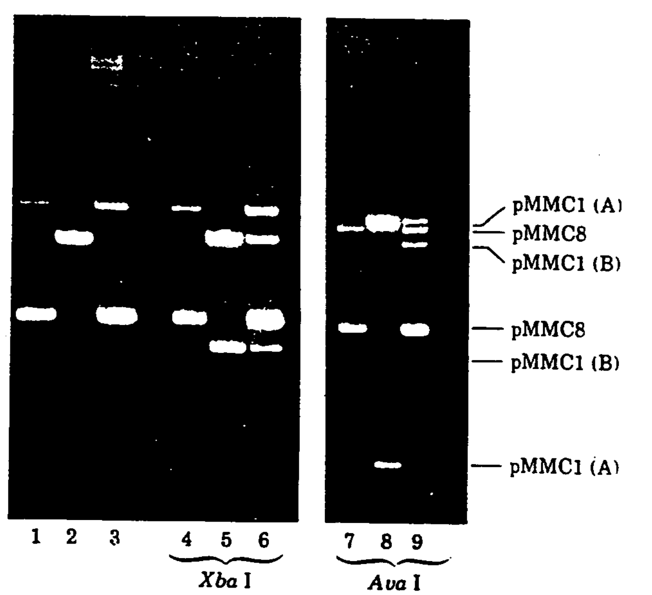

FIG. 5. FLP-promoted inversion of pMMC1 sequences. Agarose gel electrophoresis and restriction digests were as described. Lanes: 1, pMMC8 DNA; 2, pMMC1 DNA; 3, pMMC8/1 DNA; 4, *Xba* I-digested pMMC8; 5, *Xba* I-digested pMMC1 DNA; 6, *Xba* I-digested pMMC8/1 DNA; 7, *Ava* I-digested pMMC8 DNA; 8, *Ava* I-digested pMMC1 DNA; 9, *Ava* I-digested pMMC8/1 DNA. Bands in lane 9 are identified; A and B denote A and B forms of the 2-μm plasmid sequence within pMMC1.

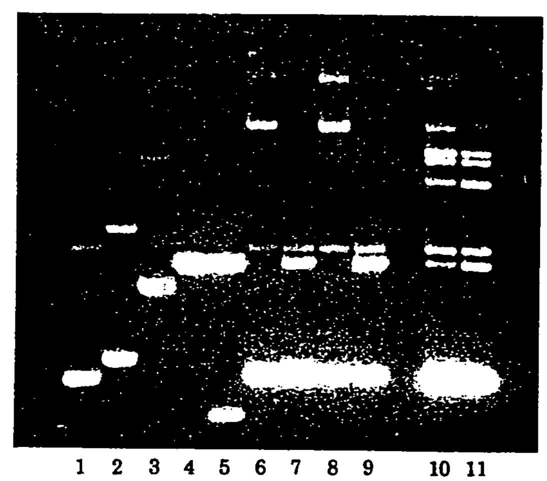

FIG. 6. FLP-promoted deletion of pMMC3 sequences. Agarose gel electrophoresis and restriction digests were carried out as described. Lanes: 1, pMMC8 DNA; 2, pMMC10 DNA; 3, pMMC3 DNA; 4, *Xba* I-digested pMMC10 DNA; 5, *Xba* I-digested pMMC3 DNA; 6, pMMC8/10 DNA; 7, *Xba* I-digested pMMC8/10 DNA; 8, pMMC8/3 DNA; 9, *Xba* I-digested pMMC8/3 DNA; 10, pMMC8/3 DNA, 0.8 μg digested for 5 min with 5 units of *Xba* I; 11, pMMC8/3 DNA, 0.8 μg digested for 10 min with 5 units of *Xba* I.

served for pMMC8/3 DNA was very similar to that observed for pMMC8/10. Cleavage of pMMC8/3 yielded not the two bands expected for pMMC3 but only the band corresponding to pMMC10. This result indicates that early after cotransformation, deletion events occurred within the pMMC3 molecules present.

In a reaction that is the molecular equivalent of the FLP-promoted inversion described above, these events would yield two small plasmids. One of these would be equivalent to pMMC10 and retain the tet^R elements of pMMC3. The other would lack an *E. coli* replication origin and would be lost. This experiment demonstrates that the FLP protein expressed by pMMC8 can promote deletion events in *trans* as well as the inversion and insertion events already demonstrated. Two partial digests of pMMC8/3 with *Xba* I also are shown in Fig. 6, revealing a number of intermediate bands. It is possible that some of these represent catenated species. Deletion events within pMMC3 multimers might be expected to yield catenated products by the considerations of Mizuuchi *et al.* (15), adding to the complexity of the gel patterns.

DISCUSSION

The site-specific recombination system of the yeast 2-μm plasmid was successfully transferred to *E. coli*. The new *E. coli* system is identified with the yeast 2-μm plasmid system by a dependence on the presence of both the *FLP* gene (expressed) and the FLP recombination site. The evidence for this conclusion relies on observation of the recombination event itself. This event requires the recombination site from the 2-μm plasmid because (*i*) restriction analyses of *in vivo* reaction products are in all cases consistent with recombination involving only these sites, and (*ii*) destruction of sequences within the site which are known to be required for the event in yeast prevents the reaction. The requirement for expression of the *FLP* gene is implied from the observation that recombination is observed only in the presence of plasmids in which the *FLP* gene has been placed at or near the position normally occupied by the *cro* gene relative to the bacteriophage λp_R (pMMC6 or pMMC8). In the absence of pMMC6 or pMMC8, plasmids containing 2-μm plasmid recombination sites (pMMC3, pMMC1, and pMMC10) are stably maintained as unrecombined, monomeric species.

The *FLP* gene in pMMC6 or pMMC8 should be temperature inducible. All of the experiments described were carried out by using cells grown at 30°C, so that the recombination observed resulted from the basal levels of FLP protein present when λp_R was repressed by cI857 repressor. The amount of FLP protein present under these conditions has not yet been determined. There is potential, however, for significant amplification of the levels of FLP protein through temperature induction. Thus, the plasmids pMMC6 and pMMC8 represent a potentially highly enriched source of FLP protein. Use of *E. coli* for this purification will avoid also the protease problems common to yeast extracts (16).

The results presented here permit several additional conclusions. (*i*) FLP protein is the active agent in this recombination event. (*ii*) The FLP protein acts efficiently in *trans*. (*iii*) No other proteins or factors specific to yeast are required. It is possible that an *E. coli* protein takes the place of a required yeast function, but it is at least as likely that FLP protein acts alone. (*iv*) FLP protein can promote all three types of site-specific recombination: deletions, inversions, and insertions. This is in contrast to the resolvase system, which appears to be highly specific for deletion events (17). This versatility is not an artifact of the *E. coli* system. FLP-promoted deletion and insertion events have been observed in yeast (1, 18). In addition, multimers of the 2-μm plasmid are prevalent in populations of this DNA isolated from yeast (19). (*v*) FLP-promoted recombination does not depend upon any feature of DNA structure that is unique to yeast. In yeast, 2-μm plasmid circle DNA is packaged by a normal complement of core histones into chromatin-like nucleosomal DNA (20, 21). It is possible that histones or other yeast proteins regulate this recombination event in yeast; the *E. coli* system may owe its efficiency, in part, to their absence.

The apparent presence of tetrameric plasmid species in preparations of pMMC4 DNA may reflect a low level of expression of FLP protein in cells transformed with this plasmid. The nature of the protein produced by pMMC4 and the level at which it is present are questions that have not yet been addressed.

No statement can be made at this time about the rate of FLP-promoted recombination or the relative efficiency of the three types of recombination observed. A more complete characterization of the FLP protein and its reactions awaits the purification of the protein.

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